CHARACTERIZATION OF *salA*, *syrF*, AND *syrG* REGULATORY NETWORKS INVOLVED IN PLANT PATHOGENESIS BY *Pseudomonas syringae* pv. *syringae* B728A

A Dissertation

by

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ABSTRACT

Pseudomonas syringae pv. *syringae* B728a, causal agent of brown spot on bean, is an economically important plant pathogen that utilizes extracellular signaling to initiate a lifestyle change from an epiphyte to a pathogen. LuxR regulatory proteins play an important role in the transcriptional regulation of a variety of biological processes involving two-component signaling, quorum sensing, and secondary metabolism. Analysis of the B728a genome identified 24 LuxR-like proteins, three of which are *salA*, *syrF*, and *syrG* located adjacent to the syringomycin gene cluster. All three proteins exhibit domain architecture that placed these LuxR-like proteins into a subfamily of LuxR's associated with regulation of secondary metabolism in *Pss* B728a. The transcriptional start sites of *salA*, *syrG*, and *syrF* were located 63, 235, and 498 bp upstream of the start codons, respectively, using primer extension analysis. The predicted -10/-35 promoter region of *syrF* and *syrG* was confirmed using site-directed mutagenesis and GFP reporters that showed there were conserved promoter sequences observed around the -35 promoter region. It has been established that SalA binds to the promoter of *syrF*, therefore these conserved promoter sequences serve as the putative binding site for SalA. Deletion mutants of *salA*, *syrF*, and *syrG* failed to produce syringomycin and displayed reduction of virulence on bean. QRT-PCR analysis results revealed that both *syrG* and *syrF* are highly expressed in the apoplast indicating that they encode important transcriptional regulators of genes critical to the plant-pathogen interaction. Additionally, this report showed that *syrG* and *syrF* are important

transcriptional regulators of syringomycin biosynthesis genes, but are not involved in the regulation of virulence genes that reside outside of the *syr-syp* gene cluster. Overexpression analysis and GFP reporters identified SyrG as an upstream transcriptional activator of *syrF*, where both SyrG and SyrF activate promoters of syringomycin biosynthesis genes. This study demonstrates that the interaction between SalA, SyrG, and SyrF for the regulation of syringomycin is complex requiring further investigation.

DEDICATION

I dedicate this work to my family and my loving husband, Rudolfo Diaz Jr. Without their patience and support; none of this would have been possible.

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I would like to thank my major advisor, Dr. Dennis Gross, and my committee members, Dr. Kolomiets, Dr. Pierson, and Dr. Shim for their support and guidance throughout the course of my academic studies and scientific research. In addition, I also would like to express my gratitude to the PLPM faculty, staff, and graduate students at Texas A&M University. They were a continuous source of encouragement and offered valuable advise on my research on multiple occasions.

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CHAPTER I

INTRODUCTION

Pseudomonas syringae is a δ-proteobacterium that is responsible for a number of plant diseases with economic importance worldwide. Originally, *Pseudomonas syringae* was isolated from diseased lilac (*Syringa vulgaris*) in 1902 by van Hall (1). Since then, *Pseudomonas syringae* has been isolated from diseased tissue on a broad range of plant species. Currently, the species is divided into pathogenic variants (pathovars), which differ in host range (2, 3). There are at least 50 pathovars that cause a wide range of plant diseases and variation in symptoms including leaf or fruit lesions, cankers, blasts, and galls (2-4). Due to its importance as a plant pathogen, *Pseudomonas syringae* has been the focus of considerable research, especially in regards to its epidemiology and virulence mechanisms (1). Several of the most studied strains differed from each other by host range and symptomology (4). For example, *P. syringae* pv. *syringae* B728a (*Pss* B728a) is the causal agent of brown spot on bean that results in the formation of water soaked and necrotic lesions on bean leaves and pods (5). Another strain, *Pss* B301D, which was isolated from pear, causes necrosis and cankers on cherry and other stone fruit (6). Both bacterial strains are known to have similar mechanisms of virulence; however, there are many questions that remain unanswered concerning the complex regulation of genes critical for plant pathogenesis.

Pss B728a has the ability to function as both an epiphyte and plant pathogen. The bacterium's pronounced epiphytic phase produces large bacterial populations

residing on the surfaces of bean leaves, where it persists until it utilizes extracellular signaling to initiate a lifestyle change from an epiphyte to a pathogen (7). Epiphytic growth in the phyllosphere of the plant is not uniform, where bacteria form aggregates that are essential for epiphytic survival (8). A variety of factors contribute to the bacterium's epiphytic survival and fitness; many of them are related to the production of exopolysaccharides (9) and the formation of aggregate biofilms (9, 10). Epiphytic populations provide a source of inoculum that is used to colonize the apoplast under appropriate conditions and multiply by using nutrients available in living host cells (11). During apoplastic colonization, *Pss* B728a extensively expresses genes associated with pathogenicity and virulence that include type III secretion systems, exopolysaccharides, siderophores, an ice nucleation protein, cell wall-degrading enzymes, and phytotoxins (12). The molecular basis for the switch from an epiphyte to pathogen is complex requiring the intricate interaction and regulation of multiple virulence factors, which makes *Pss* B728a an important model in the study of molecular plant pathogenesis (3).

Due to the availability of next generation sequencing, the scientific community has entered an era of genomics, which has enabled the rapid sequencing of numerous microbial genomes including *Pss* B728a (3, 13-15). This new era offers the opportunity to understand the function of bacterial organisms by not only identifying the function of unknown genes and proteins, but by resolving the regulatory mechanisms of these genes. *Pseudomonas* entered into the era of genomics over 10 years ago when the *P. aeruginosa* PA01 genome was sequenced (16, 17). Since then, the complete genomes of at least 24 different *Pseudomonas* strains and many more draft genomes have been

sequenced and made publically available, including three *Pseudomonas syringae* strains, *Pss* B728a, *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *P. syringae* pv. *phaseolicola* 1448a (http://www.ncbi.nlm.nih.gov). *Pss* B728a is one of the most commonly studied foliar pathogens and has a genome composed of one circular chromosome 6.09-Mb in size with 5,217 genes (3). When compared to the genome of *Pst* DC3000, *Pss* B728a possesses 976 unique genes on 14 genomic islands, not found in *Pst* DC3000 (3). Within this group of unique genes are LuxR-like transcriptional regulators, which have been defined as major regulators of secondary metabolism in *Pss* B728a (18-24). Three LuxR-like transcriptional regulators of specific interest in this dissertation are *salA*, *syrF*, and *syrG;* which have been implicated in virulence and syringomycin regulation (19).

The LuxR-like proteins SalA, SyrF, and SyrG exhibit a HTH DNA binding motif on the C-terminal region of the protein that is typical of LuxR regulatory proteins like FixJ and NarL, but lack a N-terminal autoinducer-binding domain and a receiver domain (19). Therefore, these LuxR-like proteins are considered to be part of a subfamily of LuxR proteins that is not completely defined (19, 22). SalA is part of a complex regulatory network that is involved in the biosynthesis, secretion, and regulation of syringomycin, syringopeptin, and syringolin (20). All genes identified to be part of the SalA regulon are absent from the genome of *Pst* DC3000. This transcriptional regulator is under the control of the *gacS/gacA* global signal transduction system, which controls expression of genes essential for plant pathogenesis (18). Also, it has been demonstrated that *salA* is required for the functional activation of both *syrG* and *syrF*

(19). Both *salA* and *syrF* are necessary for the biosynthesis of syringomycin and syringopeptin, which led to the conclusion that the regulatory networks involving syringomycin and syringopeptin overlap, but are not identical (19). Meanwhile, SalA mediates the regulation of syringomycin and syringopeptin through the regulation of SyrF (22). Protein sequence analysis also revealed that both SyrF and SyrG have somewhat similar protein sequences with 49% identity (19). The sequence similarity is significant given that SalA only has 27% and 26% identity to the protein sequence of SyrF and SyrG. The similarity of *syrF* and *syrG* may indicate similar regulatory gene targets. Previous research established that both *salA* and *syrF* are required for syringomycin production, where *syrG* gene expression is highly induced in the apoplast and is associated with virulence (12, 19). It is surmised that *syrG* plays a critical role in the regulation of genes associated with pathogenesis given that mutants of *syrG* displayed a significant reduction in virulence (19). It appears that the regulatory role of *syrG* in virulence is complex and may involve molecular mechanisms that may reside outside the *syr-syp* gene cluster.

Despite previous evidence that *salA*, *syrF*, and *syrG* have an effect on virulence and syringomycin production (19), the regulatory role of *syrG* in regards to syringomycin production, and the production of other secondary metabolites, remains unknown. It is hypothesized that the LuxR-like protein SyrG is involved in the regulation of genes essential for the pathogenic lifestyle of *Pss* B728a while under transcriptional control of SalA. This hypothesis is tested utilizing phenotypic characterization and quantitative real-time PCR analysis in an effort to identify new

components of the SyrG regulon, which is discussed in detail in Chapter II of this dissertation. Given previous evidence that mutants of *syrG* had an effect on virulence and syringomycin production (19), I also hypothesize that both *syrG* and *syrF* regulons overlap, specifically pertaining to the regulation of syringomycin production. This second hypothesis is tested in Chapter III by identifying essential promoter regions of *syrG* and *syrF*, and utilizing GFP reporter constructs to define the interactions of SalA, SyrF, and SyrG with the promoters of *salA*, *syrF*, *syrG*, and biosynthetic genes in the *syr-syp* cluster. If both *syrG* and *syrF* are involved in the regulation of syringomycin, it is expected that one or both of the LuxR transcriptional regulators may interact with the promoter regions of *syr-syp* biosynthesis genes. It is established that SalA controls the expression of both *syrG* and *syrF* (19), but it is unknown how SyrG effects the expression of *syrF* and vice versa. Does SyrG or SyrF play a role in the regulation of *syrF* and *syrG*? If not, are these two regulons independent of each other competing for the same binding site in the promoters of *syr-syp* biosynthesis genes? I believe it is important to answer these questions in order to fully understand the complex nature of *salA*, *syrG*, and *syrF* regulons in regards to virulence and the plant-pathogen interaction. The scientific study of these regulators may provide insight into host specificity, pathogenicity and the complex lifestyle of *Pss* B728a. By investigating and validating the regulatory functions of these transcriptional regulators, this research has the potential to expand our current understanding of the complex regulatory networks associated with pathogenesis.

CHAPTER II

CHRACTERIZATION OF SalA, SyrF, AND SyrG REGULATORY NETWORKS INVOLVED IN PLANT PATHOGENESIS BY *Pseudomonas syringae* pv. *syringae* B728A

OVERVIEW

LuxR regulatory proteins, found in prokaryotic organisms, play an important role in the transcriptional regulation of a variety of biological processes involving two-component signaling, quorum sensing, and secondary metabolism. Analysis of the B728a genome identified 24 LuxR-like proteins, three of which are *salA*, *syrF*, and *syrG* located adjacent to the syringomycin gene cluster. All three proteins exhibit helix-turn-helix (21) DNA-binding motif at their C-terminus and lack a defined N-terminal regulatory domain, which characterized these LuxR-like proteins into a subfamily of LuxR's associated with regulation of secondary metabolism in *Pss* B728a. Deletion mutants of *salA*, *syrF*, and *syrG* failed to produce syringomycin when compared to parental strain *Pss* B728a. The *salA*, *syrF*, and *syrG* mutants also significantly influenced virulence on bean. Quantitative real-time PCR analysis results revealed that both *syrG* and *syrF* are highly expressed in the apoplast indicating that they encode important transcriptional regulators of genes critical to the plant-pathogen interaction. Additionally, qRT-PCR analysis showed that *salA* is required for the activation of *syrF* and *syrG*, that *syrF* and *syrG* negatively regulate each other's gene expression, and that they both have an influence on genes associated with syringomycin biosynthesis. SyrF

and SyrG do not seem to have an effect on syringolin, syringafactin, alignate, levansucrase or achromobactin production. Overexpression of the N-terminal regions of SyrF and SyrG resulted in a decrease in syringomycin production of 81% and 97%, respectively. These results indicated that *syrG* may be responsible for regulating a broader range of genes involved in syringomycin production when compared to *syrF*.

INTRODUCTION

P. syringae pv. *syringae* B728a (*Pss* B728a) is an aggressive plant pathogen of bean that causes brown spot, a disease that results in the formation of water soaked and necrotic lesions on bean leaves and pods (5). The bacterium is highly adapted to its host where it has the ability to function as an epiphyte on leaf surfaces before invading apoplastic tissues as a plant pathogen. The bacterium's pronounced epiphytic phase produces large bacterial populations residing on the surfaces of bean leaves, where it persists until it utilizes extracellular signaling to initiate a lifestyle change from an epiphyte to a plant pathogen (7). Epiphytic populations provide a source of inoculum that is used to colonize the apoplast under appropriate conditions and multiply by using nutrients available in living host cells (11). During apoplastic colonization, *Pss* B728a extensively expresses genes associated with pathogenicity and virulence that include type III secretion systems, exopolysaccharides, siderophores, cell wall-degrading enzymes, and phytotoxins (3, 12).

Major virulence determinants in plant pathogenesis of *Pss* B728a are two lipopeptide phytotoxins, syringomycin and syringopeptin (2, 16, 25). Both phytotoxins target the cell membrane of its host due to the fact that they have an amphipathic lipopeptide structure

that allows them to insert into the cell membrane to form small pores that result in electrolyte leakage that ultimately results in cell death (2, 16). The phytotoxins are synthesized separately by modular nonribosomal peptide synthetases, which are encoded by the syringomycin-syringopeptin (*syr-syp*) gene clusters of *Pss* B728a (2, 16). Adjacent to the *syr-syp* gene cluster are three genes encoding LuxR-like proteins, SalA, SyrF, and SyrG (Fig. 2.1). These LuxR-like proteins have been implicated in virulence and syringomycin regulation (19).

LuxR proteins are a family of prokaryotic transcriptional regulators that are defined by having a helix-turn-helix (HTH) DNA binding motif on the C-terminus region of the protein and a N-terminus response regulatory domain (22, 26, 27). The LuxR superfamily can be grouped into four subfamilies based on domain architecture and the mechanism of regulatory activation, illustrated in Fig. 2.2 (22). The first subfamily consists of regulators that are part of a two-component sensory transduction system that are activated by the phosphorylation of an aspartate residue on the N-terminal region of the protein, typically by a transmembrane kinase. An example of this subfamily of LuxR is NarL (28, 29), which activates the nitrate reductase operon in *E. coli*. NarL is comprised of two domains, an N-terminal receiver domain that is controlled by phosphorylation and a C-terminal effector domain that elicits a physiological response. Phosphorylation occurs at the N-terminal domain to form dimers that recognize heptamer sequences in the promoter regions of gene targets (22, 28, 29). Regulators activated by *N*-acyl homoserine lactone comprise the second subfamily of LuxR proteins, which includes LuxR (22, 30), TraR (22, 31), CarR (22, 32), ExpR (33), LasR

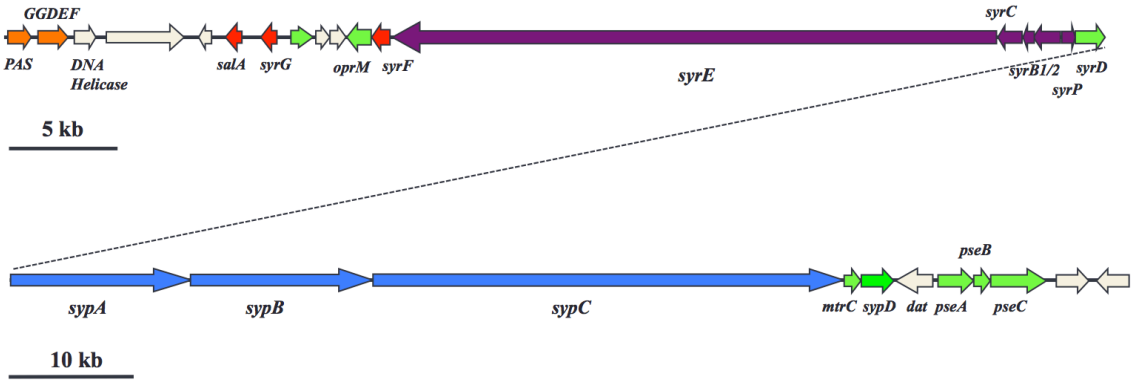

FIG 2.1. A physical map of a 132-kb genomic region of *P. syringae* pv. *syringae* B728a containing both the syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. Regulatory genes (highlighted in red) include *salA*, *syrF*, and *syrG* located on the left border region of *syr-syp* cluster. The biosynthesis genes associated with syringomycin and syringopeptin are shown as purple and blue arrows, respectively. The solid green and orange arrows represent genes involved in secretion and signal transduction.

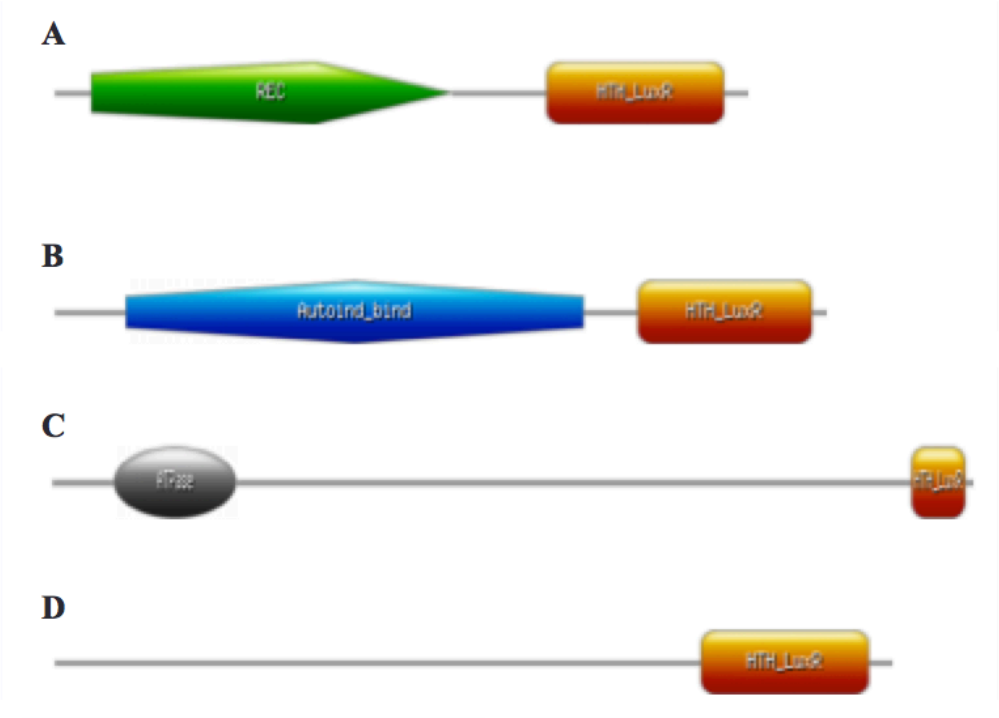

FIG 2.2. Domain organization of LuxR proteins that are classified into four sub-families based on domain architecture and mechanism of regulatory activation. **A.** GacA is a LuxR-like protein in *Pss* B728a part of a global signal transduction system characterized as having an N-terminal receiver domain activated by phosphorylated and an C-terminal HTH DNA-binding domain that is characteristic of the first sub-family of LuxR-like proteins. **B.** AhlR is part of quorum sensing system in *Pss* B728a with AhlI. It has an N-terminal auto-inducer binding domain where hexanoyl-homoserine lactone binds to activate transcription of *ahlI* and has a C-terminal HTH DNA-binding domain. This domain organization is typical of the second sub-family of LuxR's associated with quorum sensing. **C.** *Psyr_0993*, which has not been characterized in *Pss* B728a, shares homology to *malT* in *E. coli*. These genes encode a subfamily of LuxR-like proteins have an N-terminal AAA ATPase domain that requires ATP for transcriptional activation and has a C-terminal HTH DNA binding domain. **D.** SyrG, which has been implicated in virulence and syringomycin production in *Pss* B728a lacks any defined N-terminal regulatory domain and has a C-terminal HTH DNA binding domain. This domain organization is typically seen in the fourth subfamily of LuxR-like proteins, which have not been fully defined functionally. LuxR-like proteins characterized in this family of LuxR's have been associated with secondary metabolism in *Pss* B728a.

(22, 34), PhzR (35), and RhlR (36). LuxR is involved in the activation of bioluminescence related genes and is essential for quorum sensing in *Vibrio fischeri* (37). These regulators have a C-terminal HTH DNA binding domain and an N-terminal autoinducer-binding domain that interacts with acyl-homoserine lactone, which is a signaling molecule involved in quorum sensing. The third subfamily of regulators is referred to as large ATP-binding regulators of the LuxR family (LAL) (38) (39) (40). Experimentally characterized LALs include GdmRI (40), GdmRII (40), MalT (39), and PikD (38). The most studied LAL is MalT, which is the transcriptional activator of the maltose regulon in *Escherichia coli* but requires two co-factors (39) for activation (39). This subfamily of LuxR proteins is significantly different because they are relatively large in size (800 to 1,200 amino acids), contain an N-terminal ATP-binding motif, and contain a C-terminal HTH DNA binding domain (38) (39) (40). These LuxR proteins require the binding of ATP to the N-terminal region for activation. The fourth subfamily of regulators represents the simplest form of the LuxR superfamily because they harbor the typical C-terminal HTH DNA binding domain but lack a N- terminal regulatory domain. GerE was one of the first transcriptional regulators placed into this group of LuxR's (41). This regulator was involved in the transcriptional regulation of genes associated with spore formation and maturation in *Bacillus subtilis* (41). All the LuxR subfamilies are able to form dimers that interact with the promoter regions of targeted genes despite differences in the N-terminal domain region (27, 28, 42). LuxR-like proteins have been known to control transcriptional regulation of a variety of biological processes that include the production of virulence factors, biofilm formation, quorum

sensing, secondary metabolism, motility, and bioluminescence (9, 37, 43, 44).

Bioinformatic investigation of the 6.09-Mb genome of *Pss* B728a revealed 24 genes encoding LuxR-like proteins dispersed throughout the genome (Fig. 2.3). The genes *gacA*, *Psyr_1294*, *Psyr_1384*, *Psyr_1940*, *Psyr_2114*, *Psyr_3299*, *Psyr_3890*, *Psyr_4376*, *Psyr_4618*, and *Psyr_5088* encode proteins that were identified as belonging to a subfamily of LuxR's that are typically part of a two-component sensory transduction system. This subfamily is one of the largest groups of LuxR proteins found in *Pss* B728a. The LuxR-like proteins encoded on *ahlR*, *Psyr_1858*, and *Psyr_4216* are classified as belonging to the second subfamily of LuxR proteins, associated with quorum sensing. The third and smallest group of LuxR's found in *Pss* B728a belongs to the third subfamily of LuxR proteins, referred to as LAL, which includes only one LuxR protein encoded on *Psyr_0993*. *Psyr_0993* has not been functionally defined in *Pss* B728a but does encode a protein that exhibits domain architecture typical of this subfamily of LuxR proteins. The proteins that are encoded by *salA*, *sylA*, *syrG*, *syrF*, *syrR*, *Psyr_2045*, *Psyr_2578*, *Psyr_3767*, *Psyr_4266*, and *Psyr_4278* exhibit domain architecture that is typical of the fourth subfamily of LuxR proteins, which is the second largest group of LuxRs found in the *Pss* B728a genome. These transcriptional regulators seemingly play a key role in the regulation of genes associated with secondary metabolism, pathogenicity, and virulence of *Pss* B728a.

The LuxR-like proteins SalA, SyrF, and SyrG exhibit a HTH DNA binding motif on the C-terminal region of the protein that is typical of LuxR regulatory proteins like FixJ and NarL, but lack a N-terminal autoinducer-binding domain and a receiver

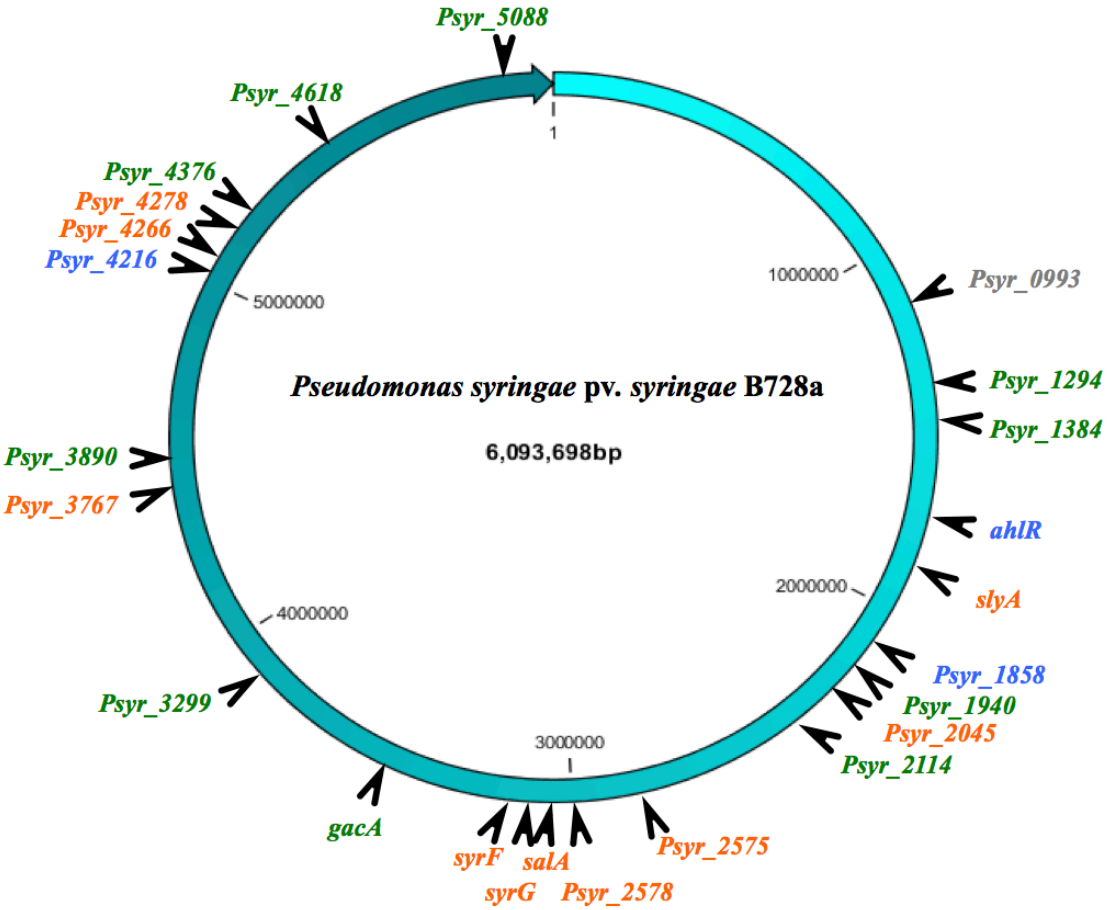

FIG 2.3. Schematic representation of LuxR-like proteins in the genome of B728a. The 6.09-Mb genome of *Pss* B728a encodes 24 LuxR-like proteins dispersed throughout the genome. Genes highlighted in green encode proteins belonging to the subfamily of LuxR's that are part of a two-component sensory transduction system. Proteins that are encoded on genes highlighted in blue belong to the second subfamily of LuxR proteins, associated with quorum sensing. The gene that encodes a protein that belongs to the third subfamily of LuxR proteins, referred to as LAL is shown in gray. Shown in orange are genes that encode LuxR-like proteins belonging to the fourth subfamily of LuxR's that lack a functionally define N-terminal regulatory domain, which includes *salA*, *syrG*, and *syrF*.

domain (19). Therefore, these LuxR-like proteins are considered to be part of the fourth subfamily of LuxR proteins that is not completely defined (19, 22).

SalA is part of a complex regulatory network that is involved in the biosynthesis, secretion, and regulation of syringomycin, syringpeptin, and syringolin (20). All genes identified to be part of the SalA regulon are absent from the genome of *Pst* DC3000. This transcriptional regulator is under the control of the *gacS/gacA* global signal transduction system, which controls expression of genes essential for plant pathogenesis (18). Also, it has been demonstrated that *salA* is required for the functional activation of both *syrF* and *syrG* (19). Both *salA* and *syrF* are necessary for the biosynthesis of syringomycin and syringopeptin, which led to the conclusion that the regulatory networks involving syringomycin and syringopeptin overlap, but are not identical (19). Meanwhile, SalA mediates the regulation of syringomycin and syringopeptin through the regulation of SyrF (22). Protein sequence analysis also revealed that both SyrF and SyrG have somewhat similar protein sequences with 49% identity (19). The sequence similarity is significant given that SalA only has 27% and 26% identity to the protein sequence of SyrF and SyrG. The similarity of *syrF* and *syrG* may indicate similar regulatory gene targets. Previous research has established that both *salA* and *syrF* genes are required for syringomycin production (19), where *syrG* gene expression is highly induced in the apoplast and is associated with virulence. It is surmised that *syrG* plays a critical role in the regulation of genes associated with pathogenesis given that mutants of *syrG* displayed a significant reduction in virulence. It appears that the regulatory role of *syrG* in virulence is complex and may involve molecular mechanisms that may reside

outside the *syr-syp* gene cluster.

Despite previous evidence that *salA*, *syrF*, and *syrG* have an effect on virulence and syringomycin production (19), the regulatory role of *syrG* in regards to syringomycin production, and the production of other secondary metabolites remains unknown. Utilizing phenotypic characterization and quantitative real-time PCR (45), the objective of this study was to identify genes under the transcriptional control of the SyrG regulon. By identifying these gene targets, current knowledge of the SalA, SyrG, and SyrF regulatory networks and their role in plant pathogenesis was further defined. In this study, it was demonstrated that *syrG* has a stronger influence on virulence and phytotoxin production than previously reported (19). This study shows that SyrG is required for virulence but is not required for the replication of *Pss* B728a *in planta*. The LuxR-like protein, SyrG, is not involved in the transcriptional regulation of known virulence genes associated with the biosynthesis of achromobactin, alginate, levansucrase, pyoverdine, syringolin, and syringafactin. Both SyrG and SyrF are required for syringomycin production with SyrG being an important transcriptional regulator of genes associated with the biosynthesis of syringomycin in *Pss* B728a.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

Bacterial strains and plasmids used in this study are listed in Table 2.1. One Shot® TOP10 chemically competent *E. coli* cells were used for cloning reactions following manufacturer's protocols (Invitrogen, Carlsbad, CA). *P. syringae* pv. *syringae* B728a strains were cultured from 20% glycerol stocks stored at -80°C onto nutrient

TABLE 2.1. Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial Strains		
Escherichia coli		
One Shot® TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
P. syringae pv. *syringae*		
B728a	Wild-type, bean pathogen; Rif^r	(3)
B728aΔ*salA*	*salA* mutant derivative of B728a, Rif^r	(46)
B728aΔ*syrF*	*syrF* mutant derivative of B728a, Rif^r	This study
B728aΔ*syrG*	*syrG* mutant derivative of B728a, Rif^r	This study
B728aΔ*syrF*Δ*syrG*	*syrF* and *syrG* mutant derivative of B728a, Rif^r	This study
B728aΔ*gacS*	*gacS* mutant derivative of B728a, Rif^r	(47)
Plasmids		
pE2602	pENTR/D-TOPO 6.80 kb region carrying *syrG*, Km^r	This study
pE2607	pENTR/D-TOPO 6.62-kb region carrying *syrF*, Km^r	This study
pKD13	Template plasmid containing FRT-flanked *nptII*	(48)
pLVCD	Gateway destination vector for mating with *P. syringae*; pBR322 derivative with *mob* genes from RSF1010; Tc^r Ap^r Cm^r	(49)
pLV2602	pLVCD carrying *syrG*; Tc^r Ap^r	This study
pLV2607	pLVCD carrying *syrF*; Tc^r Ap^r	This study
pLV2602-FP	pLVCD carrying upstream and downstream regions of *syrG* fused to *nptII*; Tc^r Ap^r Km^r	This study
pLV2607-FP	pLVCD carrying upstream and downstream regions of *syrF* fused to *nptII*; Tc^r Ap^r Km^r	This study
pPROBE-KT'	Promoter-probe vector with pVS1/p15a replicon and *gfp* reporter, Km^r	(50)
pPKT::*syrG*	pPROBE-KT' carrying *syrG* along with 752-bp upstream; Km^r	This study
pKT::*syrF*	pPROBE-KT' carrying *syrF* along with 1.3-kb upstream; Km^r	This study
pMEKm12	*E. coli* and *P. syringae* pv. *syringae* overexpression vector, Km^r	(51)
pMK::*syrG*	pMEKm12 carrying the *syrG* gene in-frame fused to *malE*; Km^r	This study
pMK::*syrG*583	pMEKm12 carrying 583-bp of the *syrG* N-terminal region fused to *malE*; Km^r	This study
pMK::*syrF*	pMEKm12 carrying the *syrF* gene in-frame fused to *malE*; Km^r	This study
pMK::*syrF*583	pMEKm12 carrying 583-bp of the *syrF* N-terminal region fused to *malE*; Km^r	This study
pRK2073	Helper plasmid; Sp^r Trm^r	(52)

broth-yeast extract (NBY) (53), or on King's B (KB) (54) at 26°C. Bioassays for syringomycin were grown on hrp-inducing minimal medium (HMM) agar (55, 56). The following antibiotic concentrations (μg/ml) were added to media: rifampicin, 100; kanamycin, 75; tetracycline, 20; ampicillin, 100; gentamycin, 5; spectinomycin, 100.

General DNA manipulations

For methodologies that involve the use of Gateway cloning technology (57 and regulatory aspects of lambda site specific recombination), targeted genes were PCR amplified and cloned into the pENTR/D-TOPO vector following the manufacturer's protocols (Invitrogen). Recombination between pENTR constructs and Gateway destination vectors was performed employing the use of LR clonase in accordance with the manufacturer's protocol (Invitrogen). Plasmids were introduced into *E. coli* by chemical transformation or electroporation (58). Plasmids were incorporated into *P. syringae* pv. *syringae* by tri-parental mating utilizing the helper plasmid pRK2073 (52). Complementation of *Pss* B728a derivative mutants was achieved by the electroporation of the complement construct. Standard PCR procedures and cycling conditions were used (47, 55).

Restriction enzymes, and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Phusion High-Fidelity DNA polymerase was purchased from Thermo Scientific Inc. (Waltham, MA). In-Fusion® HD cloning kit was purchased from Clontech Laboratories (Mountain View, CA). The design and purchase of oligonucleotides was acquired using PrimerQuest and OligoAnalyzer applications of Integrated DNA technologies (Coralville, IA). The oligonucleotide sequences are listed

TABLE 2.2. Primers used for PCR amplification

Name	Sequence (5' – 3')	Source
pE2602F	CACCGAACAGCCTTGTGCAGCGAG	This study
pE2602R	GTGAAACATTAACGTGCTCC	This study
pE2607F	CACCACCAGGTAGGTCGCAATGAC	This study
pE2607R	GTGGTGACGACCAAGGTTCT	This study
pLV2602-3F	CCGCGGGCCAGCCTCCACCGCAGAGCGTTG	This study
pLV2602-5R	GTGCACTACACATTTGCCCCCATGGCGTTA	This study
pLV2607-3F	GAGCTCACAGATCAATTGGCCCCTGGCTGT	This study
pLV2607-5R	CTCGAGATGTCCATTCCTGAAGATCAGAAG	This study
pLV2602KmF	CAAATGTGTAGTGCACGTGTAGGCTGGAGCTGC TTCGAAG	This study
pLV2602KmR	GGAGGCTGGCCCGCGGATTCCGGGGATCCGTCG ACCTGCA	This study
pLV2607KmF	GAATGGACATCTCGAGGTGTAGGCTGGAGCTGC TTCGAAG	This study
pLV2607KmR	ATTGATCTGTGAGCTCATTCCGGGGATCCGTCG ACCTGCA	This study
2602F	TCGGCTGGAGACTAACGCCA	This study
2602R	GCTCTGCGGTGGAGGCTGGC	This study
2607F	ATTGCACGCAACTTCTGATCT	This study
2607R	CCAGGGGCCAATTGATCTGT	This study
pKT2602F	GAATTCGAGCTCGCTCACGGTATTCCCCGCTCA ATG	This study
pKT2602R	GTCGACGGATCCTCACGCTATTTGAGATACGCC TGT	This study
pKT2607F	GAATTCGAGCTCTCGCCCTCGCCAACCTCTGGA AAG	This study
pKT2607R	GTCGACGGATCCTCATTCTGCGCCTATCATCCAT TT	This study
pMK2602F	GTCGACGGATCCATGGAAGCCAATCTACAAATG ACG	This study
pMK2602R	GTCGACTCTAGATCACGCTATTTGAGATACGCC TGT	This study
pMK2602-583R	GTCGACTCTAGACTGGCTCAGCTTTTCGTTGAAT TC	This study
pMK2607F	GTCGACGGATCCATGAACCGACAAGTGAATGCC AAA	This study
pMK2607R	GTCGACTCTAGATCATTCTGCGCCTATCATCCAT TT	This study
pMK2607-583R	GTCGACTCTAGAGTACTTGTACTCAATGGGAAC CGA	This study

in Table 2.2.

Bioinformatic analysis

Protein sequences were retrieved using the *Pseudomonas* Genome Database (59). The Conserved Domain Database at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/) was used to identify conserved domains of protein sequences. Additionally, database searches were performed using a Basic Local Alignment Search Tool (BLAST) to identify homologous sequences SyrG and SyrF in pseudomonad genomes. A clustalW alignment of homologous protein sequences was generated using the CLC Genomics Workbench (V5.5, CLC Bio.) (55).

Construction of markerless deletion mutants in *Pss* B728a

For targeted deletion mutants in *Pss* B728a, the gene of interest (GOI) along with 3 to 4-kb of flanking DNA was PCR amplified using Phusion® high fidelity polymerase (ThermoScientific). The purified PCR product was cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen) and transformed chemically into *E. coli* One Shot® TOP10 cells. LR clonase II (Invitrogen) was used to carry out recombination between the pENTR construct and the *Pseudomonas* suicide vector, pLVC-D (49).

Site directed mutagenesis occurred by linearization of the pLVC-D plasmid (pLVC-D:flank-GOI-flank) using inverse PCR with primers that exclude the GOI and purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). A linear kanamycin cassette flanked by the FLP recognition target sites, was amplified from pKD13 plasmid using primers with 15 bp extensions that were homologous to regions adjacent to the GOI (48). The kanamycin cassette was cloned into the purified

linearized pLVC-D construct using the In-Fusion® HD cloning kit (Clontech) according to the manufacturer's protocol and chemically transformed into *E. coli* One Shot® TOP10 cells for confirmation of the construct. The resulting pLVC-D construct (pLVC-D:flank-*nptII*-flank) was moved into *Pss* B728a by triparental mating with the helper plasmid pRK2073 (52). Colony PCR and qRT-PCR was used to confirm double recombination of the kanamycin cassette into *Pss* B728a, replacing the GOI. The kanamycin marker was later removed by the introduction of the pBH474 vector carrying the FLP recombinase gene. FLP recombination resulted in the loss of the *nptII* marker, giving markerless deletion mutant in *Pss* B728a. The Suc^s pBH474 plasmid was cured from B728a deletion mutant cells by culturing in NBY + 5% sucrose liquid medium.

Construction of complementing and overexpressing plasmids

For the complementation of B728a derivative mutants a copy of the targeted gene and the predicted promoter region was PCR amplified from *Pss* B728a with a *Bam*HI and *Sac*I restriction enzyme sited on each end of the PCR product using primers listed in Table 2.2. The PCR product was digested with *Bam*HI and *Sac*I. Additionally, the broad-host-range promoter-probe vector, pPROBE-KT' was digested with *Bam*HI and *Sac*I (50). Digested PCR products and vector were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Purified digested products were quantified using micro-spectrophotometry (Nano-Drop Technologies, Inc.). Ligation of the vector and insert was performed using T4 DNA ligase (New England Biolabs) and chemically transformed into *E. coli* One Shot® TOP10 cells (Invitrogen) for confirmation of

construct. Both constructs, pPROBE-KT':*syrF* and pPROBE-KT':*syrG*, were introduced into B728a derivative mutants by electroporation.

The overexpression of SyrF, SyrG, and their respective truncated proteins missing portions of the C-terminal region were cloned into the expression vector, pMEKm12 (51). For *syrF*, the targeted gene, and 583-bp of the N-terminal sequence was PCR amplified from *Pss* B728a with *Bam*HI and *Xba*I restriction enzyme sites flanking the PCR product. For *syrG*, the targeted gene, and 583-bp of the N-terminal sequence was PCR amplified from *Pss* B728a with *Bam*HI and *Xba*I restriction enzyme sites flanking the PCR product. The PCR products and the expression vector, pMEKm12, were digested with *Bam*HI and *Xba*I. Digested PCR products and vector were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Purified digested products were quantified using micro-spectrophotometry (Nano-Drop Technologies, Inc.). Ligation of the vector and insert was performed using T4 DNA ligase (New England Biolabs) and chemically transformed into *E. coli* One Shot® TOP10 cells (Invitrogen) for confirmation of construct. Overexpression constructs were introduced into *Pss* B728a by electroporation.

Pathogenicity assays

The ability of derivative mutants (B728a*ΔsalA*, B728a*ΔsyrF*, B728a*ΔsyrG*, and B728a*ΔsyrFΔsyrG*) to cause disease and multiply *in planta* was evaluated by vacuum infiltration on 2-week old Blue Lake 274 (Burpee Seeds, Warminster, PA) bean plants (*Phaseolus vulgaris* L.) and 4-week old *N. benthamiana*. The method for vacuum infiltration was described previously (47, 55). *Pss* B728a was used as a positive control

and *Pss* B728a*ΔgacS* served as the negative control. Each strain was evaluated on at least three plants of each species, with two biological replicates.

To evaluate the ability of derivative mutants to replicate *in planta*, population analysis was performed for B728a, B728a*ΔsalA*, B728a*ΔsyrF*, B728a*ΔsyrG*, B728a*ΔsyrFΔsyrG*, and B728a*ΔgacS* on Day 0 and Day 3 after vacuum infiltration of bean plants. From each infiltrated plant, a trifoliate leaf was detached and infiltrated tissue was removed using the bottom of a sterile 2 mL microcentrifuge tube (Bio Plas Inc., San Franscisco, CA). A total of 10 leaf discs were removed per leaf and rinsed with sterile deionized water. The leaf discs were ground using a sterile mortar and pestle with Silwet Phosphate Magnesium Buffer (SPM) (55). Serial dilutions were prepared with SPM buffer and plated on KB agar with appropriate antibiotics followed by incubation at 26°C for 48 h. Colonies were counted and calculated as CFU per squared cm.

Syringomycin assays

The production of syringomycin by *Pss* B728a and derivative mutant strains were evaluated using a bioassay previously described (25) for syringomycin production on HMM agar. Bacterial strains were grown overnight in 2 ml NBY at 26°C with shaking at 180 rpm. Cells were washed and resuspended in sterile deionized water to OD_{600} = 0.3 (~2 x 10^8 CFU/ml), and 5 µl aliquots of bacterial suspension were spotted on HMM. After an incubation period of 3 days at 26°C the plates were lightly sprayed with a cell suspension of *Geotrichum candidum* strain F-260 using a sterile chromatography sprayer. After 24 h, quantification of syringomycin production was

determined by measuring the diameter of inhibition zones and compared to the parental strain of *Pss* B728a. This experiment was repeated in triplicate.

RNA isolation

Bacterial strains were cultured overnight with shaking at 26°C in 5 ml of liquid NBY medium. Cells were harvested by centrifugation, washed and resuspended in sterile deionized water to a concentration of approximately 2 x 10^8 CFU per ml. Cell suspensions (100 µl) were spread onto HMM agar and incubated at 26°C for 48 h. Total RNA was purified using an RNeasy Mini Kit along with the RNAprotect reagent following the manufacturer's protocol (Qiagen Inc., Valencia, CA). RNA samples were treated with TURBO™ DNase (Ambion, Austin, TX) to remove residual DNA. The RNA was tested for DNA contamination using RT-PCR where RNA is used as the template with no reverse transcription reaction. The RNA quality and quantification was evaluated utilizing an Agilent 2100 Bioanlyzer (Agilent Technologies, Inc.), selecting samples with RNA Integrity Number (RIN) above 8.0 (55).

For qRT-PCR analysis, selected RNA samples were converted to cDNA by reverse transcription using Super Script Vilo™ cDNA Synthesis kit (Invitrogen) as described by Greenwald et al. (55), and diluted to 10 ng/µl. Reverse transcription was performed with the following temperature cycle: 10 min at 25°C, 60 min at 42°C, and 5 min at 85°C.

qRT-PCR analysis

To determine the effect of *salA*, *syrF*, and *syrG* deletion mutants on the expression of genes associated with syringomycin biosynthesis, epiphytic fitness and

secondary metabolism, qRT-PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System with SYBR® Select Master Mix (Invitrogen). For each 20 µl reaction the following was used: 10 µl SYBR® Select Master Mix, 8.20 µl nuclease-free water, 0.4 µl of both the forward and reverse primers (200 nM), and 1 µl of template cDNA (10 ng/µl). Primers used for qRT-PCR analysis as listed in Table 2.3, with primers specific for *recA* and *16s-rRNA* internal control genes that were used to normalize gene expression(20). For each primer pair, the linearity of detection was confirmed to have a correlation coefficient of at least 0.98 (r^2>0.98) over the detection area by measuring a 5-fold dilution curve with cDNA generated from bacterial RNA. Conditions for qRT-PCR involved an incubation temperature of 95°C for two minutes, followed by 40 cycles involving 3 seconds at 95°C and 30 seconds at 60°C. A melting curve analysis was used for each qRT-PCR reaction to validate that a single primer product was amplified.

Data was analyzed using the comparative C_t method (60), where an increase or decrease of transcript levels is determined by comparing the C_t values of the samples of interest to the C_t values of a control sample. Fold change in gene expression was calculated using the following equation: $2^{-\Delta\Delta Ct} = [(C_{t\ \text{gene-of-interest}} - C_{t\ \text{internal control}})$ Treated sample $- (C_{t\ \text{gene-of-interest}} - C_{t\ \text{internal control}})$ Untreated sample] (60). A 2 fold or more change in C_t for the sample of interest when compared to the control sample was considered to be significant (47). A decrease in fold change was computed by taking the negative inverse of the fold change value (60).

TABLE 2.3. Primers used for qRT-PCR analysis

Name	Sequence (5' – 3')	Source
16sF	CTTCGGTACGCCTGGACA	(47)
16sR	CTTCGGTACGCCTGGACA	(47)
2578F	AACATGTCAATGCCCTGCAATCGG	This study
2578R	ACGGTATTGACCTTGAGTGCGAGT	This study
3983F	CGCTGGTATCGACACCTTTAT	This study
3983R	CAGTGGAACGACTGACTGATAG	This study
acsAF	ATTTGAATGCATCCTTTCCGGCCC	This study
acsAR	CTGATTGAAATCAAGCCGCTGGCA	This study
acsBF	TGCCGGGTCTGGACATGATTCTTG	This study
acsBR	ATTACCAGCGGGCAGCGTCTC	This study
acsCF	GTTTGACGTGTTTGCGCCATCGTA	This study
acsCR	TCATTGCGGCCTGTATGTTTGCAG	This study
acsDF	GAATTCGCCAGTCGTTGCTCTAC	This study
acsDR	GCTCGACGCGCTGCCACATC	This study
algAF	GTTATCTTGTCAGGCGGTAGTG	This study
algAR	TCAACGCCAGGAACTGTTTAG	This study
hrpAF	AGGGCATCAACAGCGTAAA	This study
hrpAR	ATCGCCTTTGCTGATGCT	This study
hrpEF	TGCTTGCCAAACGCAGTATTACC	This study
hrpER	TGACGTTGCGCGTCAGTCAGAATA	This study
hrpLF	CTATCAGGAAAGCTGGGAAGAC	This study
hrpLR	GCAATCGATGGCCTCTATGA	This study
hrpZF	TCCTGAAACCGAGACGACTGG	(47)
hrpZR	GACCGTTGCGCATCAGTTCCTC	(47)
lsc-1F	ACTGGTGAAGAGTGGGAAATAC	This study
lsc-1R	TTGCCGTCCTGAAAGACATAG	This study
pslDF	GTTTACCGCCAACTCCATCTA	This study
pslDR	CCTTGACGGTGCCGATATAA	This study
pvdLF	CACCTACGCACTGGATGAAA	This study
pvdLR	AGTGATCAGTGGCCAGAAAC	This study
pvdSF	GCCGGAAATCTCGCATATCA	This study
pvdSR	CGGTACATCTCGAACGCATAA	This study
recAF	CTTCGGTACGCCTGGACA	(47)
recAR	ACACCGCCCCTCACACCA	(47)
salAF	TGAACTGCACCCTCATGCATCTCT	This study
salAR	CGATATGCTGCTTCACAATGGGCA	This study
syfAF	GTATGTGCTCGACGCTTATGA	This study
syfAR	GTCTTCGGCAGGTTCAGATAG	This study
sylAF	CCATCGGCAAGACCTGTAAT	This study
sylAR	TGGTTCTGACACCCAACTTC	This study
sypAF	ACGCGAAAGGACCAACTAC	This study
sypAR	CTCAAGGCGGTCTGCATATAA	This study

TABLE 2.3. Continued

Name	Sequence (5' – 3')	Source
sypBF	GACCGTGCATGTGACCTATTA	This study
sypBR	CCACAGGTATCGAGCAGATAAA	This study
sypCF	TTCGGAAACCACCACCTATTC	This study
sypCR	AGCAGGTAGAACTGCGTATTG	This study
syrB1F	ATATCGTCTCTGCGCGTATTG	This study
syrB1R	GTGTAGATGATGTAGGCAGTGG	This study
syrB2F	AATGGTTGCCTGCAGTTCATTCCC	This study
syrB2R	TCCTTATCGATCTGCAACTGGCGA	This study
syrCF	GGTTATCGACCGCACATCTAAT	This study
syrCR	CACTGTCGCCCAGATTGTAA	This study
syrDF	GGTGTTCTCTGACTTCCATCTG	This study
syrDR	CCTCGATCTTGACCTTGTCTTC	This study
syrEF	CATGTCACCCTGGAACTGATAC	This study
syrER	GTGCGACAAACTCCCGATAA	This study
syrPF	CTATCGCTCAACGCCGTATC	This study
syrPR	TGCTCGCAGAAGAACCATT	This study
syrrFF	ATGAACTGCTCTTGACGCAGAGT	This study
syrFR	TGAGTACAAGTACCTGCAACGCGA	This study
syrGF	ATACGCCTGTCATCCACTTGACCA	This study
syrGR	AGCTGCACGTCAAGAACAGCTCTA	This study
syrRF	CACATGCAGGACCCGTTGTTGATT	This study
syrRR	AGGCTCTTGAGCAAGGACAACTCT	This study

RESULTS

SyrG and SyrF are highly expressed in the apoplast of bean

Analysis of the 6.09 Mb *Pss* B728a genome identified 24 LuxR-like proteins dispersed throughout the genome shown in Fig. 2.3. Some of these proteins have been implicated in virulence and secondary metabolism in *Pss* B728a. To identify the LuxR-like proteins that are important to the plant-pathogen interaction, qRT-PCR analysis was used to determine transcript abundance of the genes identified as LuxR-like proteins in the apoplast of bean when compared to parental strain B728a in conditions conducive for *hrp* gene expression. The results indicated that in *Pss* B728a, both *syrG* and *syrF* are the most highly expressed LuxR genes in the apoplast of bean relative to HMM liquid medium (Fig. 2.4). The relative expression of *syrG* and *syrF* when compared to other luxR genes indicate that SyrG and SyrF proteins are involved in the transcriptional regulation of genes that potentially are critical to plant pathogenesis.

SalA, SyrF, and SyrG are novel LuxR transcriptional regulators with homologs found exclusively in *Pseudomonas syringae* genomospecies 2

An investigation of the *Pss* B728a genome identified 24 LuxR-like regulatory proteins, three of which are *salA, syrG*, and *syrF* that are located adjacent to the syringomycin gene cluster (Fig. 2.1). A conserved domain search of *salA, syrG*, and *syrF* using NCBI Conserved Domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/) confirmed the presence of a conserved helix-turn-helix DNA binding motif. The HTH DNA binding motif on the C-terminal

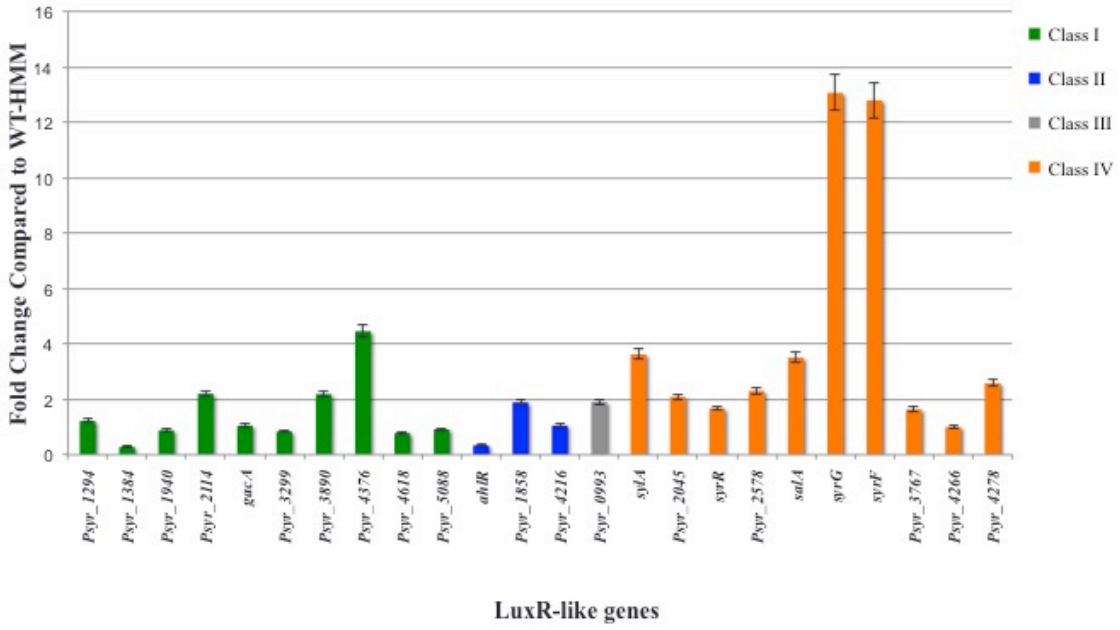

FIG. 2.4. Expression analysis in the apoplast of bean of genes encoding LuxR-like proteins in *Pss* B728a. The genes that encode proteins that are classified in the first subfamily of LuxR (Class I) are shown in the green and are typically associated with two component signal transduction systems. Shown in blue are genes that encode LuxR-like proteins implicated in quorum sensing based on domain architecture. Psyr_0993, which is shown as grey is the only gene that encodes a protein characterized as a LAL or LuxR-like proteins that require ATP for activation. The final subfamily of LuxR-like proteins are encoded on genes shown in orange bars, which lack an N-terminal regulatory domain and is associated with secondary metabolism. Out of all 24 LuxR-like proteins found in the genome of *Pss* B728a, the genes encoding SyrG and SyrF are the most highly expressed in the apoplast when compared to HMM liquid medium. The values are represented as the average fold change of three technical replicates of three biological samples. Gene expression was normalized to the *16s-rRNA* and *recA* internal control genes. Vertical bars indicate standard errors of the average values over triplicate runs.

region of the proteins is typical of LuxR regulatory proteins, but they lacked an N-terminal autoinducer-binding domain and receiver domain. BLAST analysis was also performed on SalA, SyrF, and SyrG protein sequences to determine the degree of conservation in pseudomonad strains. It was observed that all three regulatory proteins are exclusively found in *Pseudomonas syringae* genomospecies 2, with the C-terminal region being highly conserved as shown in Fig. 2.5 with regulatory protein SyrG.

Mutation strategy used to generate *syrG* and *syrF* deletion mutants in *Pss* B728a

The generation of a clean deletion mutant of *syrG* and *syrF* in *Pss* B728a was achieved by using the mutation strategy outlined in Fig. 2.6 and described in Materials and Methods. The *syrG* and *syrF* gene (Psyr_2602 and Psyr_2607) sequences were deleted from the B728a genome, and confirmed by colony PCR and qRT-PCR. The Km^r marker was removed from the derivative mutants using FLP (48), and strains lacking *Psyr_2602*, *Psyr_2607*, and both *Psyr_2602* and *Psyr_2607* were named B728aΔ*syrG*, B728aΔ*syrF*, and B728a*ΔsyrGΔsyrF*, respectively. The bacterial strains B728aΔ*syrG*, B728aΔ*syrF*, and B728a*ΔsyrGΔsyrF* displayed colony morphologies and growth curve patterns similar to parental strain B728a.

SalA, SyrF, and SyrG influence virulence of *Pss* B728a on bean plants

The bacterial strains B728a*ΔsyrG*, B728a*ΔsyrF*, and B728a*ΔsyrGΔsyrF* were significantly reduced in virulence relative to the parental strain *Pss* B728a shown in Fig. 2.7. The *salA* mutant failed to produce watersoaked necrotic lesions typical of *Pss* B728a. The *salA* mutant was comparable to B728a*ΔgacS* in regards to virulence by

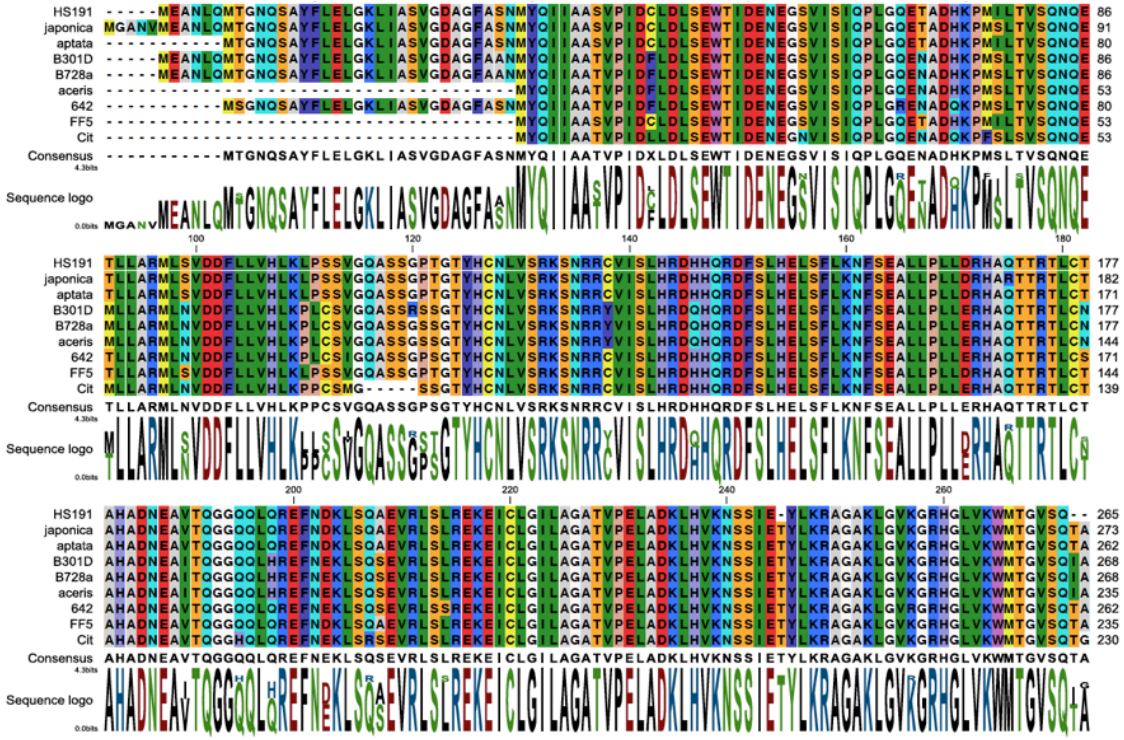

FIG. 2.5. Sequence conservation of the SyrG regulatory protein in *Pseudomonas*. SyrG is conserved only in *Pseudomonas syringae* genomospecies 2 and not in *Pst* DC3000 genomospecies 1. There is also a high level of conservation observed in the C-terminal region of the protein where there is a HTH DNA binding motif known to interact with the promoter regions of targeted genes. The sequence of SyrG in *Pss* B301D, a closely related strain to *Pss* B728a, is one amino acid different from the sequence in *Pss* B728a.

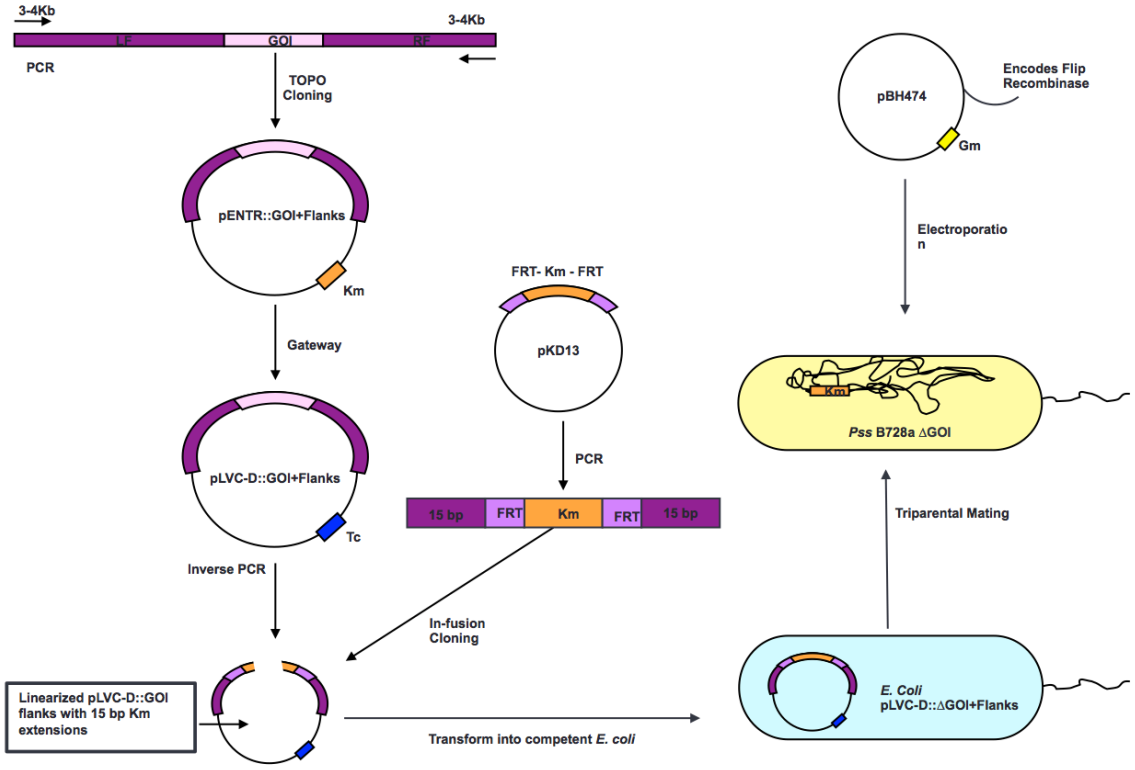

FIG. 2.6. Diagram of the strategy used to generate site-directed deletion mutants of *syrG* and *syrF* genes in *Pss* B728a. This procedure is described in Materials and Methods.

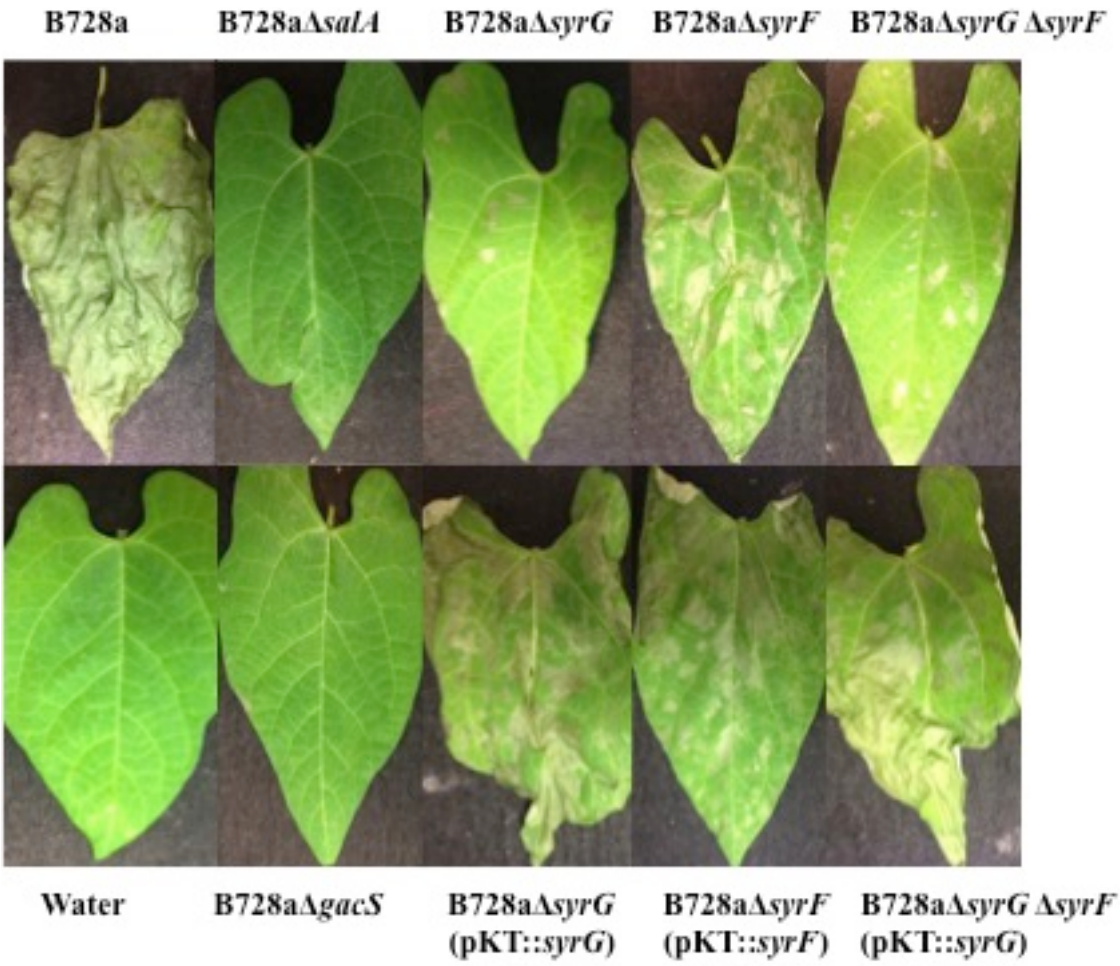

FIG. 2.7. Pathogenicity assays to evaluate the contribution of *syrG* and *syrF* to virulence on bean. Bean leaves were inoculated by vacuum infiltration with bacterial suspensions containing 10^7 CFU/cm^2 of either B728a, B728a*ΔsyrG*, B728a*ΔsyrF*, B728a*ΔsyrGΔsyrF*, B728a*ΔgacS*, B728a*ΔsyrG* (pKT::*syrG*), B728a*ΔsyrF* (pKT::*syrF*), or B728a*ΔsyrGΔsyrF* (pKT::*syrG*). Plants were maintained at room temperature in a growth chamber for 72 h. This experiment was performed twice, and representative results are shown.

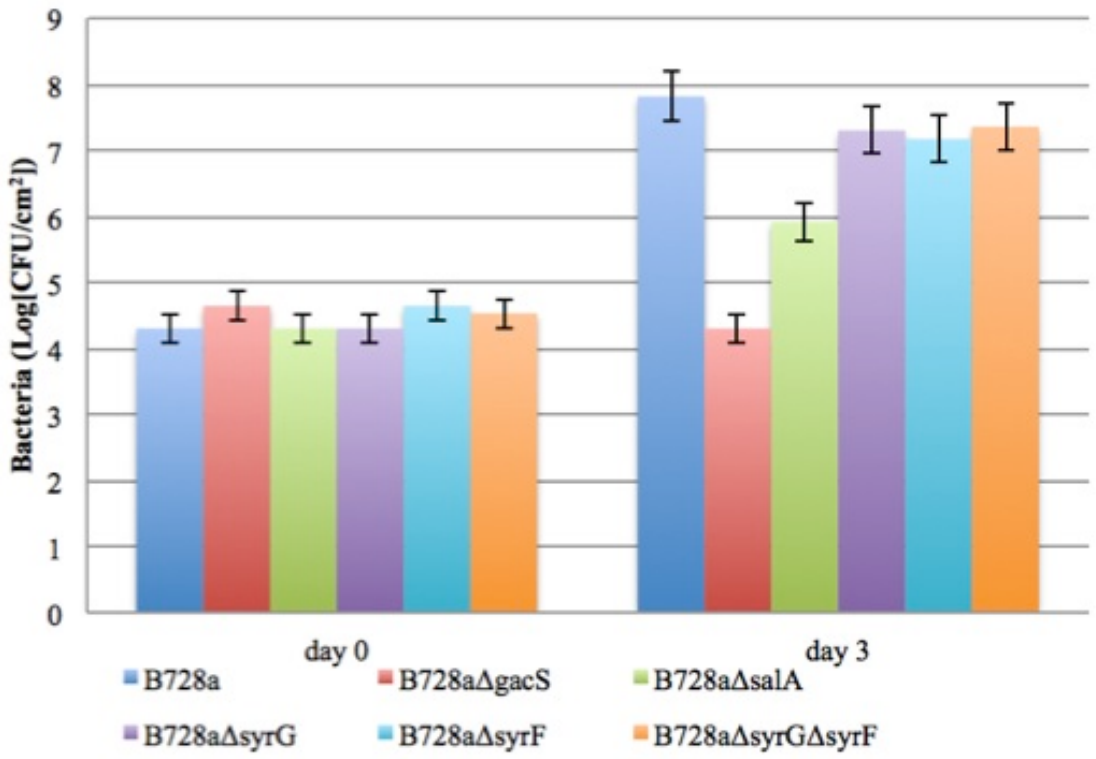

FIG. 2.8. *In planta* population counts of *Pss* B728a and mutant derivatives. Bacterial populations were monitored over a 3-day period in terms of log of CFU/cm^2 of the leaf surface. The values are represented as the average of three technical replicates of two biological samples. Vertical bars represent the standard error the average values.

lacking the ability to produce necrotic lesions and cause disease on bean. Similar to the *salA* mutant, B728a*ΔsyrG* was reduced in virulence by approximately 80% when compared to the parental strain *Pss* B728a. Mutants of *syrG* displayed small, non-spreading lesions that averaged 2 mm in diameter on bean leaves. In contrast, the *syrF* mutant was able to produce large necrotic lesions, but displayed approximately 50% reduction in virulence on bean. The double deletion mutant of *syrG* and *syrF* exhibited disease symptoms comparable to the *syrG* mutant. Virulence of *syrG* and *syrF* derivative mutants was partially restored *in trans* by complementation of *syrG* and *syrF* shown in Fig. 2.7.

Bacterial populations of infected bean plants were monitored over a 3-day period. At 3 days post-inoculation, bacterial titers for parental strain *Pss* B728a was 6.5 x 10^7 CFU/cm^2, while B728a*ΔsalA,* B728a*ΔsyrG* and B728a*ΔsyrF* were 2.0 x 10^6 CFU/cm^2, 3.2 x 10^7 CFU/cm^2 and 1.5 x 10^7 CFU/cm^2, respectively (Fig. 2.8). B728aΔ*gacS*, which fails to produce disease on bean, grew to 2.2 x 10^4 CFU/cm^2 3 days post-inoculation. The bacterial population of B728a*ΔsyrG* and B728a*ΔsyrF* were not significantly different from the parental strain *Pss* B728a, indicating that the *syrG* and *syrF* genes are not required for multiplication *in planta*. In contrast, B728a*ΔsalA* displayed a 10-fold reduction in bacterial titers compared to parental strain B728a. B728a*ΔsalA* is able to replicate *in planta*, but at a reduce rate when compared to B728a. In the case of B728a*ΔgacS*, the bacterium remains viable but is limited on its ability to multiply *in planta*.

Deletion mutants of the *salA*, *syrF*, and *syrG* genes in *Pss* B728a affect syringomycin production

The bioassay used to evaluate the influence deletion of *salA*, *syrG*, and *syrF* has on syringomycin production was determined by measuring zones of antifungal activity to *Geotrichum candidum* as compared to parental strain *Pss* B728a grown on HMM agar (Fig. 2.9). All the deletion mutants, including the double mutant *Pss* B728a*ΔsyrFΔsyrG*, displayed no measurable antifungal activity toward *G. candidum*. Antifungal activity toward *G. candidum* was partially restored when B728a mutant derivatives were complemented *in trans* with the vector pPROBE-KT' carrying an intact copy of the *syrF* or *syrG* gene.

Overexpression of N-terminal truncated proteins of SyrG and SyrF has an effect on syringomycin production

To demonstrate that the HTH DNA binding domain of SyrG and SyrF are essential for binding to *syr-syp* promoters, the N-terminal and C-terminal regions of SyrG and SyrF were overexpressioned in *Pss* B728a. The overexpression of the C-terminal regions onf SyrG and SyrF had no effect on syringomycin production, however, overexpression of the N-terminal regions of SyrG and SyrF in *Pss* B728a resulted in the dramatic reduction of syringomycin zones of inhibition to *Geotrichum candidum* from 16 mm to 0.5 mm and 3 mm, respectively (Fig. 2.10). The overexpression of the N-terminal regions of SyrG and and SyrF resulted in a 97% and 81% reduction in syringomycin production, which can be attributed to nonfunctional heterodimers formed between wild-type proteins and the truncated proteins. These nonfunctional heterodimers

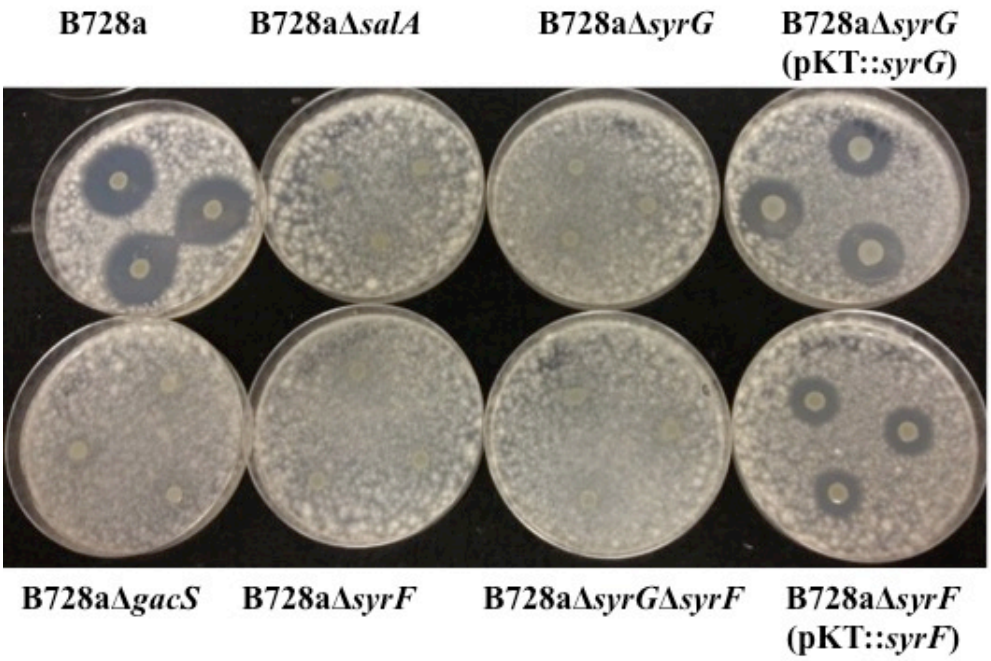

FIG. 2.9. Bioassy to evaluate syringomycin production in parental strain B728a and derivative mutants. Bacterial strains were grown on HMM for 4 days. Plates were oversprayed with *Geotrichum candidum* and incubated 24 h at 26°C. Zones of inhibition were measured to determine the effect each mutant had on syringomycin production. The experiment was repeated in triplicate.

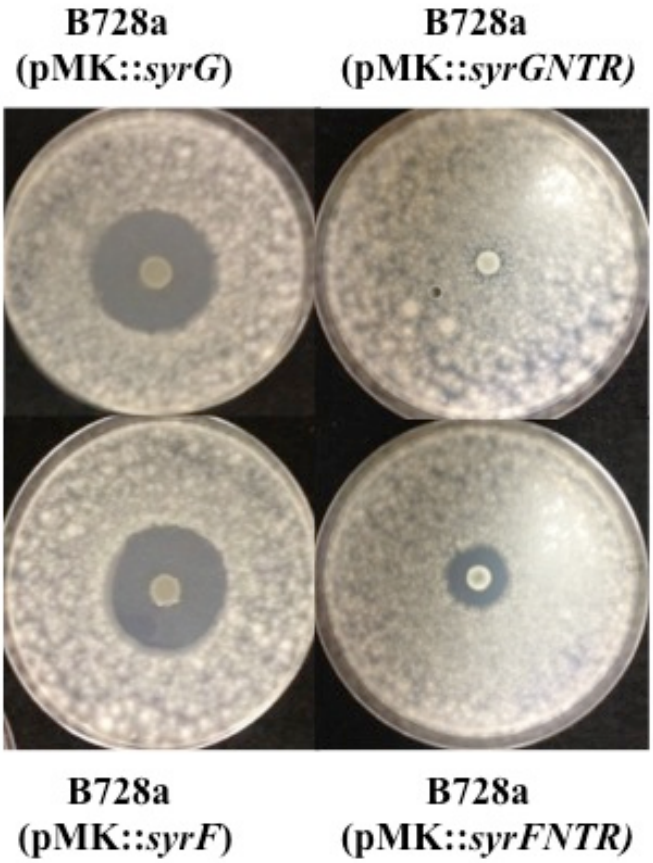

FIG. 2.10. Effect of overexpression of N-terminal region (NTR) of SyrG and SyrF on syringomycin production in *Pss* B728a. Bacterial strains were grown on HMM for 4 days. Plates were oversprayed with *Geotrichum candidum* and incubated 24 h at 26°C. Zones of inhibition were measured to determine the effect each mutant had on syringomycin production. The experiment was repeated in triplicate.

lack the ability to properly bind to *syr-syp* promoters, which is essential for the transcriptional activation genes required for syringomycin production. The truncation of SyrG displayed the greatest reduction in syringomycin production, which is comparable to the overexpression of N-terminal truncated proteins of SalA in B301D (22).

The effect of *syrG* and *syrF* deletion mutants on genes associated with virulence

Previous experiments have established both *syrG* and *syrF* have an influence on syringomycin production and virulence in *Pss* B728a. Quantitative real-time PCR (45) was used to identify the effect *syrG* and *syrF* deletion mutants have on genes associated with syringomycin production and virulence. A total of 23 genes were evaluated using qRT-PCR that included genes involved in the biosynthesis of syringomycin, syringopeptin, achromobactin, alginate, levansucrase, syringolin, syringafactin, and pyoverdine. Both *syrG* and *syrF* had an effect on the expression of syringomycin biosynthesis genes (Fig. 2.11). A deletion mutant of *syrG* resulted in a 4- to 11-fold decrease in transcript abundance of *syrB1*, *syrB2*, *syrC*, *syrD*, *syrE* and *syrP*. In regards to *syrF*, the deletion mutant resulted in a 2- to 12-fold decrease of transcript abundance of syringomycin biosynthesis genes. The results also indicated that both SyrG and SyrF are involved in the transcriptional regulation of genes associated with syringomycin production. Mutants of *syrG* and *syrF* did not appear to have an effect on the expression of genes associated with achromobactin, alginate, levansucrase, syringolin, syringofactin, or pyverdine biosynthesis (data not shown). Both *syrG* and *syrF* failed to have an effect on known virulence genes outside of the *syr-syp* gene cluster.

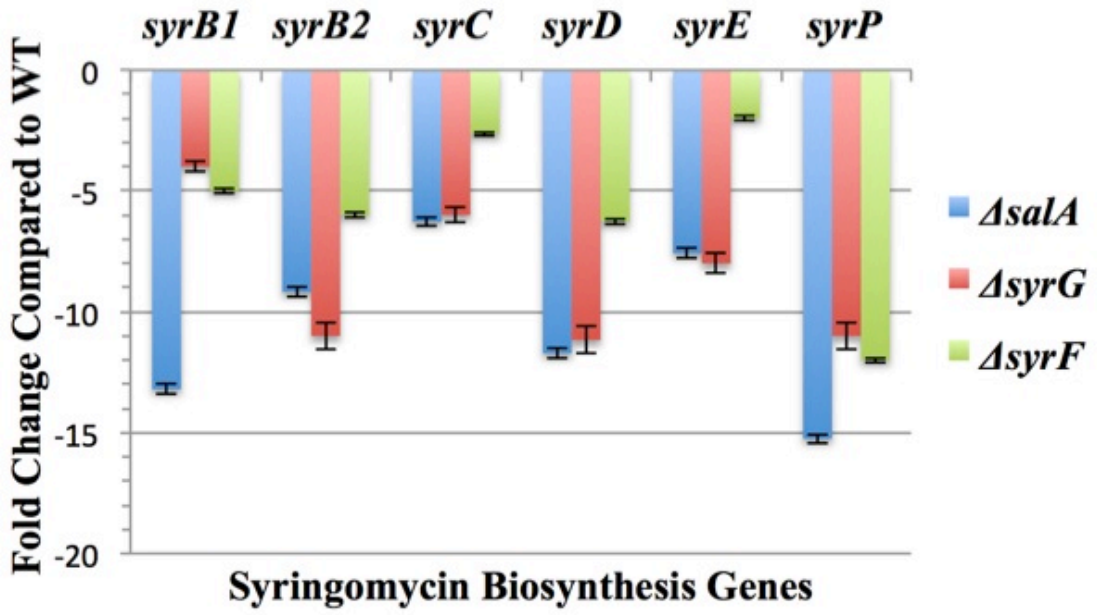

FIG. 2.11. Quantitative real-time PCR analysis of syringomycin biosynthesis genes in *ΔsalA*, *ΔsyrG*, and *ΔsyrF* mutants of *Pss* B728a. The values represent the average fold change in gene expression from parental strain Pss B728a; the results are the averages of three technical replicates from three biological samples grown in HMM liquid media. Gene expression levels were normalized to *16s-rRNA* and *recA* internal control genes, and vertical bars indicate standard errors of the average values over triplicate runs. Negative values indicate a decrease in transcript abundance by taking the negative inverse of a fold change value less than 1.

The effect of *syrF* and *syrG* deletion mutants on LuxR-like homologs in *Pss* B728a

To determine the effect *syrF* and *syrG* mutants have on LuxR-like homologs in *Pss* B728a, qRT-PCR analysis was performed using primers specific for *salA*, *syrG*, *syrF*, *sylA*, *syrR*, and *Psyr_2578*. SylA and SyrR are LuxR-like proteins that have been implicated in the regulation of syringolin and syringofactin (12, 21, 46). Results from qRT-PCR analysis show that *syrG* and *syrF* require a functional *salA* gene for activation (Fig. 2.12). Mutants of *salA* displayed a 3.6-, and 4.51-fold decrease in transcript abundance of *syrG* and *syrF*, respectively. Mutants of *syrG* displayed 27-fold increase in transcript abundance of *syrF*, and mutants of *syrF* displayed a 20-fold increase in transcript abundance of *syrG*. Data obtained from qRT-PCR analysis indicated that both *syrG* and *syrF* negatively regulate expression of each other's gene. SyrG and SyrF did not have an effect on the expression of the LuxR-like genes *sylA*, *syrR*, and *Psyr_2578* indicating they are not part of the SyrG or SyrF regulatory networks.

DISCUSSION

The genome of *Pss* B728a is relatively large in size (6.09-Mb), and encodes 24-LuxR-like proteins. These LuxR-like proteins have been associated with a variety of biological processes that includes quorum sensing, virulence, and secondary metabolism in *Pss* B728a (12, 18-21, 46, 47, 61). The superfamily of LuxR-like proteins may be categorizied into four subfamilies based on domain architecture and mechanism of

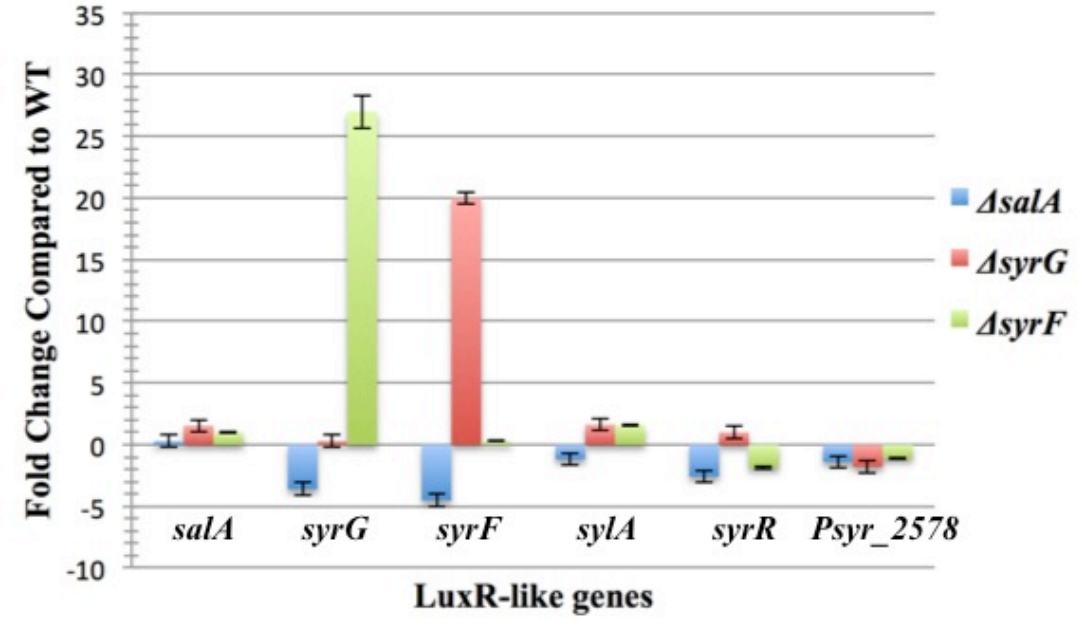

FIG. 2.12. Quantitative real-time PCR analysis of LuxR-like genes in *ΔsalA*, *ΔsyrG*, and *ΔsyrF* mutants of *Pss* B728a. The values represent the average fold change in gene expression from parental strain Pss B728a; the results are the averages of three technical replicates from three biological samples grown in HMM liquid media. Gene expression levels were normalized to *16s-rRNA* and *recA* internal control genes, and vertical bars indicate standard errors of the average values over triplicate runs. Negative values indicate a decrease in transcript abundance by taking the negative inverse of a fold change value less than 1.

regulatory activation shown in Fig. 2.2. Located adjacent to the syringomycin gene cluster, *salA*, *syrG*, and *syrF*, which are three LuxR-like proteins that are classified into a subfamily of LuxR proteins not completely characterized. These proteins lack a defined N-terminal regulatory domain, but possess a highly conserved C-terminal HTH DNA binding domain. The HTH DNA binding motif is known to interact with the promoter elements of targeted regulatory genes to induce or repress transcription (21, 22). Sequence analysis of SalA, SyrG, and SyrF showed that these LuxR-like proteins are closely related to FixJ and NarL. These LuxR-like proteins belong to a subfamily of LuxR-like proteins that are part of a two componenet signal transduction systems that require phosphorylation of the N-terminal receiver domain for activation (28, 29, 41). However, SalA, SyrG, and SyrF share the greatest sequence homology to the LuxR-like protein GerE that is involved in the regulation of spore formation in *B. subtilis* (41). The GerE protein also lacks an N-terminal regulatory domain (41). LuxR-like proteins that lack a N-terminal regulatory domain are part of a LuxR subfamily that is not completely defined that may act as transcriptional activators and repressors. Analysis of the crystal structure of GerE revealed that it is comprised of four alpha helices, of which the central pair forms a HTH DNA-binding motif in the C-terminal region of the protein.(41). LuxR-like proteins exhibiting a similar domain organization have been associated with secondary metabolism in *Pss* B728a. For example, SlyA activates the transcription of *slyB* and *sylC* which are involved in the biosynthesis of syringolin (21). *SyrR* and *Psyr_2578* encode two LuxR-like proteins that are located adjacent to *syfA* and *syfB*. Both *syfA* and *syfB* are required for biosynthesis of syringafactin (12, 46, 59).

Sequence analysis of SalA, SyrG, and SyrF revealed that these LuxR-like proteins are only found in *Pseudomonas syringae* genomospecies 2 with the C-terminal region displaying the highest level of conservation. The high degree of conservation observed in *Pseudomonas syringae* genomospecies 2 of these LuxR-like proteins implicate SalA, SyrG, and SyrF as important transcriptional regulators of genes associated with host specificity, pathogenicity, or the complex lifestyle of *Pss* B728a.

The importance of SalA, SyrG, and SyrF in regards to pathogenicity on bean plants was demonstrated by qRT-PCR analysis and pathogenicity assays. Quantitative real-time PCR analysis revealed that both *syrG* and *syrF* are the most highly expressed LuxR-like genes in the apoplast of bean relative to HMM liquid medium. This result indicated that both SyrG and SyrF are involved in regulation of genes important to establishing plant-pathogen interaction or pathogenesis. Consequently, pathogenicity assays showed that mutants of *salA*, *syrG*, and *syrF* displayed a significant reduction in virulence by approximately 100%, 80%, and 50%, respectively. Disease development as observed for a mutant of *syrG* was comparable to a mutant of *salA*, whereas a mutant of *syrF* produced larger necrotic lesions on bean. A double mutant of *syrF* and *syrG* in B728a displayed symptomology comparable to the mutant of *syrG*. This results indicated that *syrG* is involved in regulating a broader range of genes critical to plant pathogenesis, and that it acts upstream of the LuxR-like protein SyrF in a regulatory cascade. Mutants of *syrG* and *syrF* were able to produce bacterial populations similar to the parental strain B728a *in planta*, indicating that *syrG* and *syrF* are not required for replication in the apoplast.

Reduction in virulence seen with mutants of *syrG* and *syrF* can be attributed largely to the reduction in syringomycin and syringopeptin production. Syringomycin is considered one of the major virulence determinants of *Pss* B728a, along with syringopeptin (25). Both *syrG* and *syrF* are required for syringomycin production (shown in Fig. 2.9). Syringomycin production was partially restored when a functional copy of *syrG* and *syrF* was expressed *in trans*. Quantitative real-time PCR also showed that mutants of *syrG* and *syrF* resulted in a significant decrease in the expression of syringomycin biosynthesis genes. These results were surprising given that a previous study by Lu et. al (19) with site directed insertional mutants of *salA*, *syrF*, and *syrG* in *Pss* B301D displayed a 100%, 83%, and 40% reduction in syringomycin production when compared to the parental strain, respectively. It was hypothesized that with the insertional mutants of *syrF* and *syrG* produce truncated proteins with reduced functional activity displaying low levels of toxin production. This hypothesis was tested by overexpressing of the N-terminal regions of SyrG and SyrF in *Pss* B728a, which resulted in a significant reduction of syringomycin production (Fig. 2.10). The overexpression of the N-terminal regions of SyrG and and SyrF resulted in 97% and 81% reduction in syringomycin production, which can be attributed to the formation of a nonfunctional heterodimers unable to bind to the promoter regions of genes associated with syringomycin production. Similar results were seen in *V. fischeri* when the over expression of the N-terminal domain of LuxR displayed a reduction in luminescence (62). The amino acids between 116 and 161 in the N-terminal domain were critical for LuxR to form dimers and activate transcription of the *luxICDABE* operon(62). The

truncation of SyrG displayed the greatest reduction in syringomycin production, which is comparable to the overexpression of N-terminal region of SalA in B301D (22). Wang et. al (22) showed that the overexpression of N-terminal region of SalA and SyrF resulted in a significant decrease in expression of *syrB1:uidA* and *sypA:uidA* reporters. These results indicated that SyrG is responsible for regulating a broader range of genes involved in syringomycin and syringopeptin production when compared to SyrF.

Quantitative real-time PCR analysis was used to identify new components of the SyrG and SyrF regulon that had not been identified previously. It was revealed that *syrG* and *syrF* do not appear to affect the production or expression of alginate, achromabactin, levansucrase, syringolin, syringafactin, and pyoverdine. These results are not suprising given that a microarray study performed by Wang et. al (22) showed that genes involved in siderophore production, environmental stress, quorum sensing, global regulation, phytohormone synthesis and alginate production were not part the SyrF regulon. Both *syrG* and *syrF* failed to have an effect on known virulence genes outside of the *syr-syp* gene cluster leaving the conclusion that *syrG* and *syrF* are part of over-lapping regulons involved in the regulation of syringomycin production. In addition, my study showed SyrG and SyrF have been shown to negatively regulate the expression of each other's gene. These results indicated that the interaction between SyrG and SyrF is complex. It is believed that both SyrG and SyrF regulons overlap and as a result they are in competition for the binding and transcriptional activation of genes associated with syringomycin production.

Even though *salA*, *syrG*, and *syrF* were previously characterized, the role of syrG in regards to virulence remained undefined. Characterization of *salA*, *syrG*, and *syrF* was important to define the complex regulatory mechanism used for the expression of genes associated with virulence. SalA was established as a major regulator of virulence genes in *Pss* B728a, and is required for the activation of both SyrG and SyrF (19, 46). Both SyrG and SyrF are essential for the transcriptional activation of syringomycin biosynthesis genes. Intitally, it was believed that SyrG is responsible for the transcriptional activation of a broader range of genes associated with virulence than SyrF. However, this study did not identify virulence genes, outside of the *syr-syp* gene cluster, under the transcriptional control of SyrG. Nevertheless, my study demonstrated that SyrG is important for the transcriptional regulation of syringomycin and maybe involved in an overlapping regulon with SyrF in the regulation of syringomycin. There is a strong indication that the interactions between SalA, SyrG, and SyrF for the regulation of syringomycin may be complex, which is the topic of further study in Chapter III.

CHAPTER III

CHARACTERIZATION OF SyrF AND SyrG PROMOTER REGIONS IN *Pseudomonas syringae* pv. *syringae* B728A

OVERVIEW

Major virulence determinants in the plant pathogenesis of *Pss* B728a are two lipopeptide phytotoxins, syringomycin and syringopeptin, which are synthesized separately by modular nonribosomal peptide synthetases that are encoded by the syringomycin-syringopeptin (*syr-syp*) gene clusters. Adjacent to the *syr-syp* gene clusters are three LuxR-like proteins, SalA, SyrG, and SyrF. Previous studies implicated these proteins in the transcriptional control of genes associated with virulence and phytotoxin production. In this study, it is shown that *syrF* is transcribed in a polycistronic operon with a gene encoding an outer membrane efflux protein, *oprM*, whereas *salA* and *syrG* are transcribed as monocistronic mRNA based on reverse transcriptional PCR and bioinformatic analyses. The transcriptional start sites of *salA*, *syrG*, and *syrF* were located 63, 235, and 498 bp upstream of the start codons of *salA*, *syrG*, and *syrF*, respectively, using primer extension analysis. Assays of *syrF* and *syrG* promoter deletions that were transcriptionally fused to a *gfp* reporter, defined promoter sequences required for gene activation. Activation of both *syrF* and *syrG* promoters requires a functional *salA* gene. The predicted -10/-35 promoter region of *syrF* and *syrG* was confirmed using site directed mutagenesis, which shows there are conserved promoter sequences around the -35 promoter region. These conserved promoter

sequences may serve as the binding site for SalA. Furthermore, the interaction of SyrF and SyrG with the promoter regions of *syrB1*, *syrF*, and *syrG* was validated using GFP reporter assays. Evidence showed that both SyrG and SyrF activate promoters of syringomycin biosynthesis genes, and SyrG is an upstream transcriptional activator of *syrF*.

INTRODUCTION

P. syringae pv. *syringae* B728a is the causal agent of brown spot disease on bean (3). The bacterium grows to large bacterial populations on the leaf surface and uses extracellular signaling to initiate a change from an epiphyte to a plant pathogen (3, 8). The molecular switch from an epiphytic to a pathogenic lifestyle is complex and involves the intricate interaction and regulation of multiple virulence factors. The lipopetide phytotoxins, syringomycin and syringopeptin are considered important virulence factors that contribute to the disease development of brown spot on bean. The phytotoxins function by inserting into the cell membrane of the host to form small pores that result in electrolyte leakage and cell death (2, 16, 63). Genes responsible for the biosynthesis of syringomycin and syringopeptin are encoded on two adjacent gene clusters referred to as the *syr-syp* gene cluster (2, 16, 64). Adjacent to the *syr-syp* gene cluster are three transcriptional regulatory genes, *salA*, *syrF*, and *syrG* that were identified as encoding LuxR-like proteins (19).

LuxR-like proteins have been known to control transcriptional regulation of a variety of biological processes that include the production of virulence factors, biofilm formation, quorum sensing, secondary metabolism, motility, and bioluminescence (9, 37,

43, 44). In *Pss* B728a, these transcriptional regulators were defined as major regulators of virulence and secondary metabolism (18-24). These regulators typically have a C-terminal helix-turn-helix (21) DNA binding domain and an N-terminal autoinducer-binding domain that interacts with acyl-homoserine lactone. LuxR proteins function by forming dimers that recognize heptamer sequences in the promoter regions of gene targets (22, 28). One of the most studied regulatory proteins is LuxR, which is involved in the activation of bioluminescence related genes and is essential for quorum sensing in *Vibrio fischeri* (37). LuxR binds to a *lux* box that is located at the -42.5 position relative to the transcriptional start site of *luxI*, which activates transcription of the *lux* operon (22, 65). A similar sequence motif was identified in the promoter regions of *syr-syp* genes, termed the *syr-syp* box (23). The level of interaction seen between promoters of targeted genes to LuxR transcriptional regulators is dependent on the binding affinity of the promoters to the regulatory proteins (43).

The LuxR-like proteins SalA, SyrF, and SyrG exhibit a HTH DNA binding motif on the C-terminal region of the protein that is typical of LuxR regulatory proteins like FixJ and NarL (29, 41), but lack a N-terminal autoinducer-binding domain and a receiver domain (19). Therefore, these LuxR-like proteins are considered to be part of a novel LuxR subfamily (19, 22). Previous studies have implicated these LuxR-like proteins in virulence and the regulation of syringomycin biosynthesis genes. It was established by Wang et. al (22) that both *salA* and *syrF* form homodimers that bind to the promoter regions of *syr-syp* genes. The promoter region targeted by SyrF in the *syr-syp* gene cluster was identified as a conserved *syr-syp* box (23). Meanwhile, SalA mediates the

regulation of syringomycin and syringopeptin through the regulation of *syrF* (22). However, the precise interaction between SalA, SyrF, and SyrG and the promoter regions of *syr-syp* genes in *Pss* B728a need to be further defined.

The transcriptional organization and promoters of the *syr-syp* genes were characterized by Wang et. al (23), however essential promoter regions for the functional transcription of *syrF* and *syrG* were not identified. The objectives of this study were to elucidate the promoter regions of *syrF* and *syrG*; and define the regulatory interactions of *salA*, *syrF*, *syrG*, and the promoter region of syringomycin biosynthesis genes. These objectives were achieved by transcriptional analysis to determine the transcriptional start sites and common characteristics of the promoter regions of *syrF*, and *syrG*. Additionally, GFP reporter assays were used to determine the functional activity of promoters transcriptionally fused to *gfp* in *Pss* B728a mutant derivatives. The GFP assay further defined the regulatory interactions of SalA, SyrF, and SyrG with the promoters of *salA*, *syrF*, *syrG*, and *syrB1*. GFP reporters showed that both SyrG and SyrF activate promoters of syringomycin biosynthesis genes, and SyrG is an upstream transcriptional activator of *syrF*.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 3.1. One Shot® TOP10 chemically competent *E. coli* cells were used for general cloning reactions following manufacturer's protocols (Invitrogen). *P. syringae* pv. *syringae*

TABLE 3.1. Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial Strains		
Escherichia coli		
One Shot® TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
P. syringae pv. *syringae*		
B728a	Wild-type, bean pathogen; Rif^r	(3)
B728aΔ*salA*	*salA* mutant derivative of B728a, Rif^r	(55)
B728aΔ*syrF*	*syrF* mutant derivative of B728a, Rif^r	This study
B728aΔ*syrG*	*syrG* mutant derivative of B728a, Rif^r	This study
B728aΔ*syrF*Δ*syrG*	*syrF* and *syrG* mutant derivative of B728a, Rif^r	This study
Plasmids		
pLVCD	Gateway destination vector for mating with *P. syringae*; pBR322 derivative with *mob* genes from RSF1010; Tc^r Ap^r Cm^r	(49)
pLV2602	pLVCD carrying *syrG*; Tc^r Ap^r	This study
pLV2607	pLVCD carrying *syrF*; Tc^r Ap^r	This study
pPROBE-KT'	Promoter-probe vector with pVS1/p15a replicon and *gfp* reporter, Km^r	(50)
pPKT::*syrG*752	pPROBE-KT' carrying *syrG* along with 752-bp upstream; Km^r	This study
pPKT::*syrG*552	pPROBE-KT' carrying *syrG* along with 552-bp upstream; Km^r	This study
pPKT::*syrG*452	pPROBE-KT' carrying *syrG* along with 452-bp upstream; Km^r	This study
pPKT::*syrG*352	pPROBE-KT' carrying *syrG* along with 352-bp upstream; Km^r	This study
pPKT::*syrG*352-10	pPROBE-KT' carrying syrG along with 352-bp upstream with the potential -10 region replaced with CTGCAG; Km^r	This study
pPKT:*syrG*352-35	pPROBE-KT' carrying syrG along with 352-bp upstream with the potential -35 region replaced with CTGCAG; Km^r	This study
pPKT::*syrG*252	pPROBE-KT' carrying *syrG* along with 252-bp upstream; Km^r	This study

TABLE 3.1. Continued

Designation	Relevant Characteristics	Source
pPKT::*syrG*202	pPROBE-KT' carrying *syrG* along with 202-bp upstream; Km^r	This study
pPKT::*syrG*102	pPROBE-KT' carrying *syrG* along with 102-bp upstream; Km^r	This study
pPKT::*syrG*52	pPROBE-KT' carrying syrG along with 52-bp upstream, Km^r	This study
pPKT::*syrF*1000	pPROBE-KT' carrying *syrF* along with 1.0-kb upstream; Km^r	This study
pPKT::*syrF*800	pPROBE-KT' carrying *syrF* along with 800-bp upstream; Km^r	This study
pPKT::*syrF*600	pPROBE-KT' carrying *syrF* along with 600-bp upstream; Km^r	This study
pPKT::*syrF*600-10	pPROBE-KT' carrying *syrF* along with 600-bp upstream with the potential -10 region replaced with CTGCAG; Km^r	This study
pPKT::*syrF*600-35	pPROBE-KT' carrying *syrF* along with 600-bp upstream with the potential -35 region replaced with CTGCAG; Km^r	This study
pPKT:*syrF*500	pPROBE-KT' carrying *syrF* along with 500-bp upstream; Km^r	This study
pPKT::*syrF*252	pPROBE-KT' carrying *syrF* along with 252-bp upstream; Km^r	This study
pPKT::*syrF*202	pPROBE-KT' carrying *syrF* along with 202-bp upstream; Km^r	This study
pPKT::*syrF*152	pPROBE-KT' carrying *syrF* along with 152-bp upstream; Km^r	This study
pPKT::*syrF*102	pPROBE-KT' carrying *syrF* along with 102-bp upstream; Km^r	This study
pPKT::*syrF*52	pPROBE-KT' carrying *syrF* along with 52-bp upstream; Km^r	This study
pRK2073	Helper plasmid; Sp^r Trm^r	(52)

B728a strains were maintained on nutrient broth-yeast extract (NBY) (53), or on King's B (KB) (54)at 26°C. The following antibiotic concentractions (μg/ml) were added to media: rifampicin, 100; and kanamycin, 75.

General DNA manipulations

Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Thermo Scientific Phusion High-Fidelity DNA polymerase was purchased from Thermo Scientific Inc. (Waltham, MA). The design and purchase of oligonucleotides was acquired using PrimerQuest and OligoAnalyzer applications of Integrated DNA technologies (Coralville, IA). The oligonucleotide sequences are listed in Table 3.2. Plasmids were introduced into *E. coli* by chemical transformation or electroporation (58). Plasmids were transferred to *P. syringae* pv. *syringae* by tri-parental mating using the helper plasmid pRK2073 (52). Standard PCR procedures and cycling conditions were used.

Construction of overexpressing plasmids

The overexpression of SyrF and SyrG were cloned into the expression vector, pMEKm12 (51). For *syrF* and *syrG*, the targeted gene was PCR amplified from *Pss* B728a with *Bam*HI and *Xba*I restriction enzyme sites flanking the PCR product. The PCR products and the expression vector, pMEKm12, were digested with *Bam*HI and *Xba*I. Digested PCR products and vector were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Purified digested products were quantified using micro-spectrophotometry (Nano-Drop Technologies, Inc.). Ligation of the vector and insert

TABLE 3.2. Primers used for RT-PCR

Name	Sequence (5' – 3')	Source
salAF	AACAAAGATGCCATGGAAGG	This study
salAR	CCGTGCTGACTTTCAGATCA	This study
syrGF	ACAGGCGCTATGTCATTT CC	This study
syrGR	CCCTGTGTAATGGCTTCGTT	This study
syrFF	CCAATCCGGTATGAAAAACG	This study
syrFR	AGAGTTTTTCGGCGATCTCA	This study
oprMF	CGGTGACCAGCGGTACTTAT	This study
oprMR	ACTCGCCACCAGGCTTAGTA	This study

was performed using T4 DNA ligase (New England Biolabs) and chemically transformed into *E. coli* One Shot® TOP10 cells (Invitrogen) for confirmation of the construct. Overexpression constructs were introduced into *Pss* B728a derivative mutants by electroporation.

Operon analysis of *salA*, *syrF,* and *syrG* in *Pss* B728a using RT-PCR

RT-PCR analysis was performed to define the operons that encompass *salA*, *syrF*, and *syrG* using RNA isolated from *Pss* B728a. Primers designed for *salA*, *syrF*, *syrG*, and neighboring genes (Table 3.2) were used to identify if they were transcribed as monocistronic or polycistronic mRNA. Total RNA was prepared from *Pss* B728a by growing cells on HMM agar for 48 h at 26°C and harvesting total RNA using an RNeasy Mini Kit along with the RNAprotect reagent following the manufacturer's protocol (Qiagen Inc., Valencia, CA). RNA samples were treated with TURBO™ DNase (Ambion, Austin, TX) to remove residual DNA. The RNA was tested for DNA contamination using RT-PCR where RNA is used as the template with no reverse transcription reaction. The RNA quality and quantification was evaluated using an Agilent 2100 Bioanlyzer (Agilent Technologies, Inc.), and selecting samples with an RNA Integrity Number (RIN) above 8.0 (55).

Using approximately 100 ng of total RNA from *Pss* B728a, RT-PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System with a OneStep RT-PCR kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Reverse transcription was performed by incubating at 50°C for 30 min. After reverse transcription, RT-PCR was carried out using the following temperature

cycle: 95°C for 15 minutes, followed by 30 cycles involving 30 seconds at 94°C and 30 seconds at 55°C. After RT-PCR, amplified products were subjected to electrophoresis.

Primer extension analysis

Primer extension was performed using the Primer Extension System (Promega, Madison, WI), and a sequence marker that was created using the Sequenase Version 2.0 DNA sequencing kit following the manufacturer's instructions (Affymetrix, Santa Clara, CA). Oligonucleotides salAPE, syrFPE, and syrGPE were radiolabeled with [γ-^{32}P]ATP (Perkin Elmer, Inc., Boston, MA) at the 5' end. Primer extension was performed with 1.0 pmol of the labeled primer and 15 μg of total RNA from *Pss* B728a. Total RNA from *Pss* B728a was prepared as described previously (55). The plasmids pLV2602, and pLV2607 were used as templates to create of sequencing ladders of the upstream regions of *salA*, *syrF*, and *syrG*.

Computer analysis

Nucleotide sequences that were 100-bp upstream of identified transcriptional start sites were analyzed using the Softberry Bprom algorithm (http://linux1.softberry.com/berry.phtml) to identify putative σ^{70}-dependent promoters. These putative promoter sequences were aligned with T-Coffee (66)

Construction of GFP translational fusions and mutagenesis

To define and characterize the promoter regions of *syrF* and *syrG,* promoter fragments were PCR amplified from *Pss* B728a genomic DNA using primers listed in Table 3.3 and cloned into a *gfp* broad-host-range promoter-probe vector, pPROBE-KT', resulting in translational fusions to *gfp*. *Bam*HI and *Sac*I restriction enzyme sites

TABLE 3.3. Primers used for PCR amplification and primer extension analysis

Name	Sequence (5' – 3')	Source
salAPE	ATGCGGGAAAAGCTGTTGCATGTT	This study
syrFPE	ATCTTTGGCATTCACTTGTCGGTT	This study
syrGPE	TCCCGTCATTTGTAGATTGGCTTC	This study
pKT-salApF	GAATTCGAGCTCGCCAGCCTCCACCGCAGAGCGTTG	This study
pKT-salApR	GTCGACGGATCCTCAGACAGCTGCCTGAAACATCTC	This study
pKT-syrB1pF	GAATTCGAGCTCGAACAAACTCCTGGACCTCAGCCC	This study
pKT-syrB1pR	GTCGACGGATCCTCAGACCGCTTCGAATTTCTTGCC	This study
pKT-syrG752pF	GAATTCGAGCTCGCTCACGGTATTCCCCGCTCAATG	This study
pKT-syrG552pF	GAATTCGAGCTCGGGGCGTCGCCGATTGCTCTATCC	This study
pKT-syrG352pF	GAATTCGAGCTCTCATGTATGTCGCTGTAAACGTCG	This study
pKT-syrG252pF	GAATTCGAGCTCATGTCCTCTTATGGTTTTTGCCAA	This study
pKT-syrG210pF	GAATTCGAGCTCGGGCGCTGGCGAACCGGCCTGTAG	This study
pKT-syrG202pF	GAATTCGAGCTCGCGAACCGGCCTGTAGCGAGACGT	This study
pKT-syrG102pF	GAATTCGAGCTCCAATCAGACAGTATACTCATTACT	This study
pKT-syrGR	GTCGACGGATCCTCACGCTATTTGAGATACGCCTGT	This study
pKT-syrF1300pF	GAATTCGAGCTCTCGCCCTCGCCAACCTCTGGAAAG	This study
pKT-syrFR	GTCGACGGATCCTCATTCTGCGCCTATCATCCATTT	This study
pKT-syrPpF	GAATTCGAGCTCGACCAAAGCTCCTGTGTAATAACC	This study
pKT-syrPpR	GTCGACGGATCCTCAGGCCGGTTGCCAAACGTCGCC	This study

flanked all promoter fragments. Amplified promoter fragments were digested with *Bam*HI and *Sac*I along with the broad-host-range promoter-probe vector, pPROBE-KT'(50). Digested PCR products and vector were purified using Wizard® SV Gel and PCR Clean-Up System (Promega) and quantified utilizing micro-spectrophotometry (Nano-Drop Technologies, Inc.). Ligation of the vector and insert was performed using T4 DNA ligase (New England Biolabs) and chemically transformed into *E. coli* One Shot® TOP10 cells (Invitrogen) for confirmation of constructs. Additionally, *salA*, *syrB1*, and *syrP* promoters were cloned in pPROBE-KT'. Cloned pPROBE-KT' constructs were introduced into B728a derivative mutants by electroporation.

GFP assays

Quantitative GFP assay was performed as described by Miller et. al (50). *E. coli* cells were cultured overnight in LB with the appropriate antibiotics at 37°C with shaking. For *Pss* B728a, cells were cultured overnight in NBY with the appropriate antibiotics at 26°C with shaking. Cells were harvested, washed, and resuspended in 10 mM phosphate buffer to a concentration of 2 x 10^9 cells per mL. GFP fluorescence was measured on Tecan SpectraFluor (Tecan) at an excitation wavelength of 485 nm, and an emission wavelength of 525 nm. Intensity readings were represented by arbitrary units and normalized to a cell density of 10^9 cells per mL.

Syringomycin assays

The production of syringomycin of *Pss* B728a, and derivative mutant strains were evaluated using a bioassay previously described (25) for syringomycin production on HMM agar. Bacterial strains were grown overnight in 2 ml of NBY at 26°C with

shaking at 180 rpm. Cells were wash and resuspended in sterile deionized water to OD_{600} = 0.3 (~2 x 10^8 CFU/ml), and 5 µl aliquots of bacterial suspension were spotted on HMM. After an incubation period of 3 days at 26°C the plates were lightly sprayed with a cell suspension of *Geotrichum candidum* strain F-260 using a sterile chromatography sprayer. After 24 h, quantification of syringomycin production was determined by measuring the diameter of inhibition zones and results were compared to the parental strain of *Pss* B728a. This experiment was repeated in triplicate.

RESULTS

The *syrF* gene is in an operon with *oprM,* where *salA* and *syrG* are monocistronic mRNA transcripts

The *syr-syp* gene cluster consists of genes involved in the biosynthesis, regulation and secretion of syringomycin and syrinopeptin. These genes are organized into two operons that were defined by Wang et. al (23) in *Pss* B301D. Located adjacent to the *syr-syp* gene cluster are the LuxR-like regulatory genes *salA*, *syrF*, and *syrG*. Illustrated in Fig. 3.1, RT-PCR analysis revealed that both *salA* and *syrG* are transcribed as moncistronic mRNA with their own native promoter regions. There were no *salA-syrG* products observed with RT-PCR using primers specific to the 3' and 5' sequences of these genes, respectively (Fig. 3.1, lane 2). The genes that are organized in a polycistronic operon are *syrF* and *oprM*. A *syrF-oprM* product was obtained with RT-PCR using primers oprMF and syrFR that were specific for the 3' and 5' sequences of these genes, respectively (Fig. 3.1, lane 5).

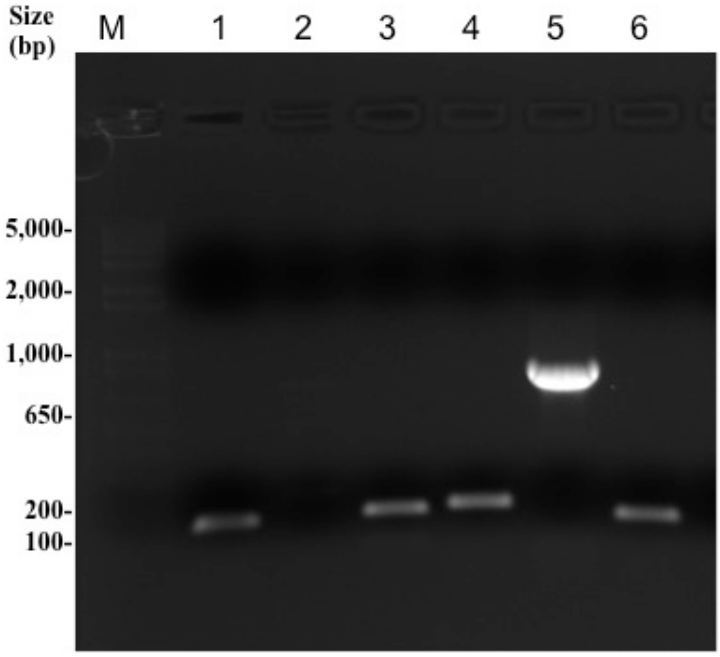

Fig. 3.1. RT-PCR analysis to identify the operons that include *salA*, *syrG*, and *syrF* genes. RT-PCR analysis was performed with total RNA isolated from *Pss* B728a growing on HMM medium for 48 h at 26°C and subjected to electrophoresis. RT-PCR products were named after the genes they encompass. **M**, 1 kb+ ladder; **1**, *salA*; **2**, *salA-syrG*; **3**, *syrG*; **4**, *syrF*; **5**, *syrF-oprM*; **6**, *oprM*.

The characterization of transcriptional start sites of *syrG* and *syrF* genes

After identifying the monocistronic and polycistronic transcripts of *salA*, *syrF*, and *syrG;* the transcriptional start sites of the respective genes were defined using primer extension analysis. For *salA*, the salAPE primer was radio-labeled and used to identify the transcriptional start site. Primer extension analysis revealed the transcription start site was at the thymine residue, which was 63 bp upstream to the translational start codon of *salA*. For *syrF*, the syrFPE primer was used to identify the transcriptional start site at the cytosine residue, which was revealed to be 498 bp upstream of the translation start codon. The syrGPE primer was used to identify the transcriptional start site at the thymine residue, 235 bp upstream of the translation start codon. The transcriptional start site of *syrG* suggests a putative promoter region sequence, $CTGAGAN_{17}TCTTAT$ (Fig. 3.2). Similarly, the transcriptional start site of *syrF* suggests a putative promoter region sequence, $TTGTTAN_{23}TGCAAC$. In addition, computer analysis of promoter sequences identified conserved sequences observed around the -35 promoter regions of *syrG* and *syrF*, illustrated by the nucleotide sequence alignment of the predicted promoter regions (Fig. 3.3). These putative promoter regions were predicted using defined transcriptional start sites and BPROM promoter prediction software (67). Both promoters share high similarity to the consensus promoter sequence of σ^{70} found in gram-negative bacteria (68).

Identification of essential *syrG* and *syrF* promoter regions

The effects of promoter deletion mutants on the expression of *syrG*::*gfp* and *syrF*::*gfp* fusions are showin in Fig. 3.4. For *syrG::gfp* fusions, deletion constructs were

salA TTGCCCACAGGACTCAAAGAATACAAAACACTTACTTATTCA**T**

syrG TCTTTTATTCTGAGACGTTGCACTGAATGTCCTCTTATGGTTT**T**

syrF ACCTTGTTACGCAGCCATCTTGCTGTGGCCCATGCAACCCATAT**C**

σ^{70} TTGACA TATAAT

-35 **-10**

Fig. 3.2. Comparison of putative promoter sequences of *salA*, *syrG* and *syrF*. Predicted promoter sequences are based on defined transcriptional start sites and using BPROM promoter prediction software. Conserved sequence motifs corresponding to -35 and -10 promoter regions are underlined and compared to a σ^{70} dependent promoter sequence. The defined promoter region of *salA* is distinctly different from the predicted promoter regions of *syrG* and *syrF*.

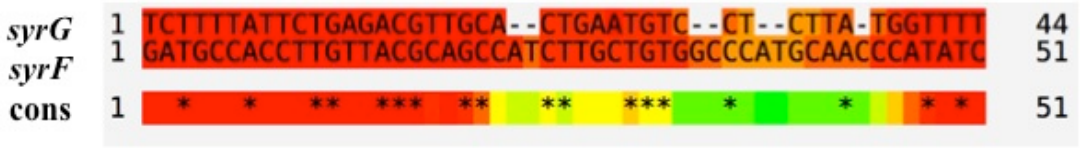

Fig. 3.3. Alignment of *syrG* and *syrF* promoter sequences in *Pss* B728a. The predicted promoter sequences of *syrG* and *syrF* were aligned using T-COFFEE and conserved sites are shown as asterisks. The color code is based on CORE index, using consistency among pairwise alignments for estimating reliability. Sequences shown in red indicate high reliability, where green is indicative of low reliability.

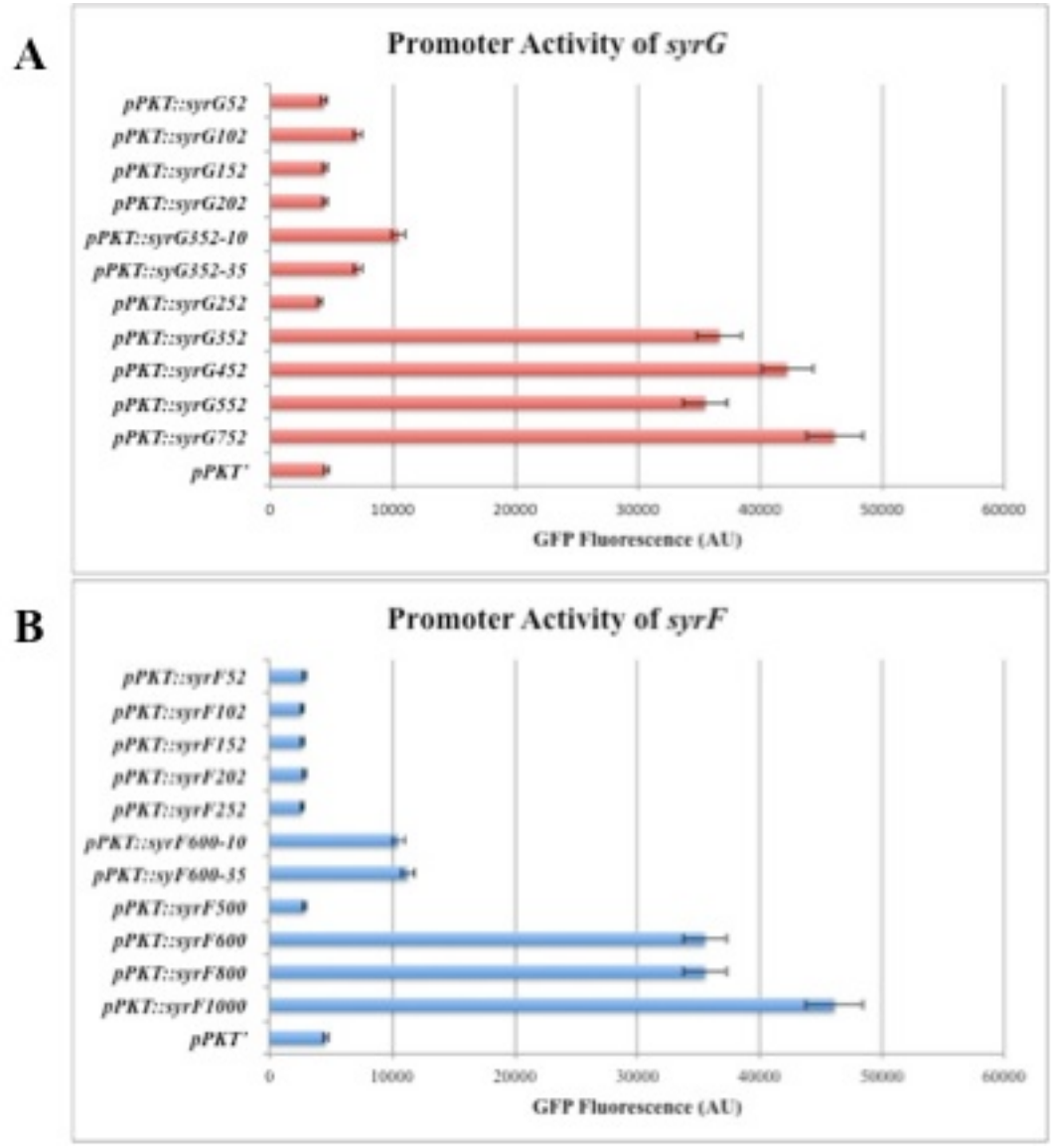

Fig. 3.4. Analysis of the promoter regions of **A,** *syrG* and **B,** *syrF* by testing the effect deletion mutants have on the expression of *syrG::gfp* and *syrF::gfp* transcriptional fusions. All *gfp* constructs were electroporated into cells of *Pss* B728a to measure GFP fluorescence (AU). All measurements were averages over three technical replicates of three biological samples. Horizontal bars represent the standard error of the average values.

generated from 752-bp, 552-bp, 452-bp, 352-bp, 252-bp, 202-bp, 152-bp, 102-bp, and 52-bp upstream of the translational start site of *syrG*. For *syrF::gfp* fusions, deletion constructs were generated from 1000-bp, 800-bp, 600-bp, 500-bp, 252-bp, 202-bp, 152-bp, 102-bp, and 52-bp upstream of the translational start site of *syrF*. Results indicated that the 100-bp region of 252- to 352-bp upstream of the start codon of *syrG* is critical for the expression of the *syrG*::*gfp* fusion. In addition, the 100-bp region of 500- to 600-bp upstream of the start codon of *syrF* is critical for the expression of the *syrF::gfp* fusion. When the predicted -10 promoter region TCTTAT of *syrG* and TGCAAC of *syrF* was substituted with CTGCAG, expression of the *syrG::gfp* and *syrF::gfp* reporters in *Pss* B728a decreased by 71% and 70%, respectively. When the predicted -35 promoter region CTGAGA of *syrG* and TTGTTA of *syrF* was substituted with CTGCAG, expression of the *syrG::gfp* and *syrF::gfp* reporters in *Pss* B728a decreased by 80% and 68%, respectively.

LuxR-type transcription regulator SalA is a positive regulator of *syrF* and *syrG* promoters

The effect deletion mutants of *salA*, *syrG*, and *syrF* has on *salA::gfp, syrG::gfp,* and *syrF::gfp* reporter gene activities was shown in Table 3.4. In the parental strain *Pss* B728a, the *salA::gfp* construct (pPKT::*salA*) displayed the relative GFP fluorescence of 43044 AU/10^9 CFU. GFP fluorescence of *salA::gfp* fusions were reduced by 76% in *salA* derivative mutant of *Pss* B728a. Deletion mutants of *syrG* and *syrF* did not significantly reduce the GFP fluorescence of *salA::gfp* transcriptional fusions. Reporter gene activities of *syrG::gfp* and *syrF::gfp* were significantly reduced by 91% and 95% in

Table 3.4. Effect of *salA*, *syrG*, and *syrF* on *salA*, *syrG*, *syrF*, and *syrB1* reporter gene activity.

Strain	Reporter	GFP Fluorescence ± SE
B728a (pKT::salA)	*salA*	43,044 ± 203
B728aΔ*salA* (pPKT::*salA)*	*salA*	10,055 ± 357
B728aΔ*syrG* (pPKT::*salA*)	*salA*	42,991 ± 425
B728aΔ*syrF* (pPKT: *salA)*	*salA*	38,106 ± 454
B728a (pKT:*syrG*)	*syrG*	45,776 ± 773
B728aΔ*salA* (pKT: *syrG)*	*syrG*	4,175 ± 237
B728aΔ*syrG* (pKT: *syrG)*	*syrG*	6,121 ± 449
B728aΔ*syrF* (pKT: *syrG)*	*syrG*	47,875 ± 389
B728a (pKT:*syrF*)	*syrF*	44,857 ± 718
B728aΔ*salA* (pKT: *syrF)*	*syrF*	2,202 ± 223
B728aΔ*syrG* (pKT: *syrF)*	*syrF*	6,163 ± 239
B728aΔ*syrF* (pKT: *syrF)*	*syrF*	7,713 ± 281
B728a (pKT:*syrB1*)	*syrB1*	47,266 ± 2,158
B728aΔ*salA* (pKT: *syrB1)*	*syrB1*	9,653 ± 626
B728aΔ*syrG* (pKT: *syrB1)*	*syrB1*	24,347 ± 522
B728aΔ*syrF* (pKT: *syrB1)*	*syrB1*	29,458 ± 345

GFP fluorescence were averaged over three technical replicates of three biological samples followed by the standard error of the averaged values.

B728a*ΔsalA* when compared to the parental strain of *Pss* B728a. These results indicate SalA is a transcriptional activator that functions upstream of *syrG* and *syrF*.

SyrG is a positive regulator of *syrF* promoters.

The effect deletion mutants of *syrG* and *syrF* have on the *syrG::gfp* and *syrF::gfp* reporter gene activities was shown in Table 3.4. The parental strain *Pss* B728a harboring the *syrG::gfp* transcriptional fusion displays a relative GFP fluorescence of 45776 AU/10^9 CFU. GFP fluorescence of *syrG::gfp* decreased by 86% in *syrG* derivative mutants of *Pss* B728a, where the GFP fluorescence *syrG::gfp* was not significantly reduced in *syrF* deletion mutants. These results indicated that SyrG is required to activate its own gene expression, but SyrF does not have an affect on the promoter activities of *syrG*. The parental strain *Pss* B728a harboring the *syrF::gfp* transcriptional fusion displayed a relative GFP fluorescence of 44857 AU/10^9 CFU. The relative GFP fluorescence of *syrF::gfp* decreased to 86% and 83% in deletion mutants of *syrG* and *syrF*, respectively. Both SyrG and SyrF are required for activation of the *syrF* gene.

Both *syrG* and *syrF* are involved in the expression of *syrB1* in *Pss* B728a.

The effect deletion mutants of *salA*, *syrG* and *syrF* have on the *syrB1::gfp* reporter gene activity is shown in Table 3.4. The parental strain *Pss* B728a harboring the *syrB1::gfp* transcriptional fusion displays a relative GFP fluorescence of 47266 AU/10^9 CFU. GFP fluorescence of *syrB1::gfp* decreased by 80%, 48%, and 38% in deletion mutants of salA, *syrG*, and *syrF*, respectively. Both SyrG and SyrF are involved in the expression of *syrB1*.

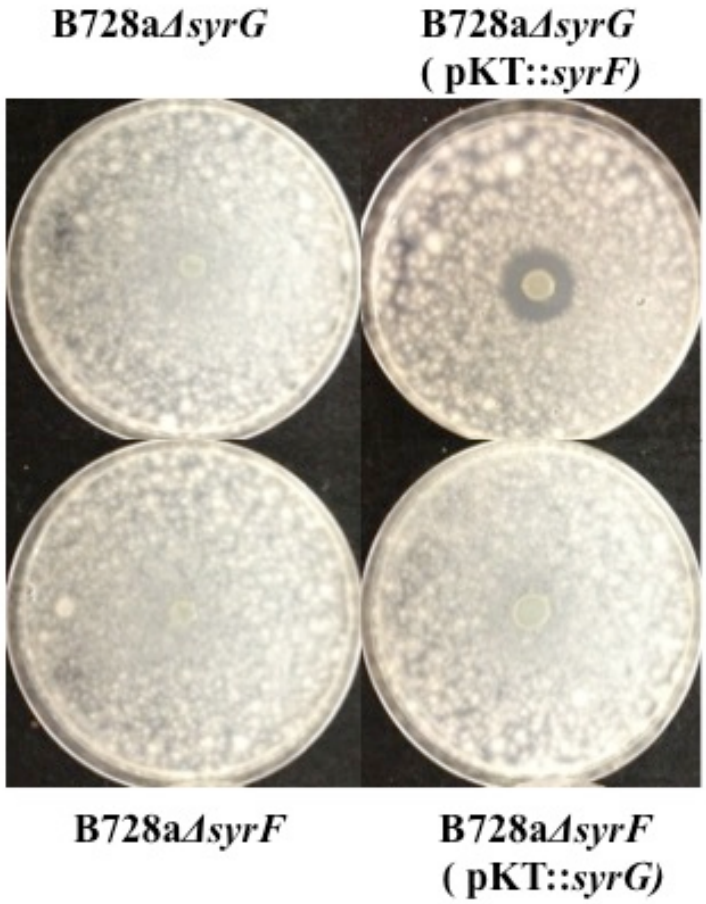

Fig. 3.5. Effects of overexpression of *syrG* and *syrF* on syringomycin production in *syrG* and *syrF* deletion mutants of *Pss* B728a. Bacterial strains were grown on HMM for 4 days. Plates were oversprayed with *Geotrichum candidum* and incubated 24 h at 26°C. Zones of inhibition were measured to determine the effect overexpression had on syringomycin production. The experiment was repeated in triplicate.

Overexpression of SyrF restores syringomycin production in *syrG* deletion mutants of *Pss* B728a.

The effect that overexpression of SyrG and SyrF have on syringomycin production in *syrG* and *syrF* derivative mutants is shown in Fig. 3.5. It was reported in Chapter II that derivative mutants of *syrG* and *syrF* in *Pss* B728a displayed a significant loss of syringomycin production when compared to the parental strain *Pss* B728a. The overexpression of SyrF had the ability to partially restore syringomycin production in *syrG* derivative mutants. Syringomycin inhibition zones increase from 0 mm to 25 mm, resulting in a 25% increase in syringomycin production when compared to a *syrG* derivative mutant. However, the overexpression of *syrG* failed to restore syringomycin production in *syrF* derivative mutants. These results indicated that SyrG is an upstream activator of *syrF*.

DISCUSSION

P. syringae pv. *syringae* B728a uses a variety of sigma factors including (ECF) sigma factors and transcriptional regulators to coordinated the expression of genes in response to environmental signals (45, 47, 55, 69). Throughout the genome of *Pss* B728a are genes that encode 24 LuxR-like proteins, some of which that have been implicated in the transcriptional regulators of genes associated with two component signaling, quorum sensing, secondoary metabolism and virulence in *Pss* B728a (12, 18-21, 61). Genes responsible for the biosynthesis of syringomycin and syringopeptin are located on two adjacent gene clusters referred to as the *syr-syp* gene cluster (2, 16, 64). Adjacent to the *syr-syp* gene cluster are three transcriptional regulatory genes, *salA*,

syrG, and *syrF* that were identified as encoding LuxR-like proteins (19). Research studies described in chapter II of this dissertation implicated *salA*, *syrG*, and *syrF* in virulence and as important transcriptional regulators of the *syr-syp* gene cluster (20, 23), where the interactions between SalA, SyrG, and SyrF in the regulation of *syr-syp* appear to be complex. In order to further define the complex regulation of *syr-syp*, the objectives of this study were to elucidate the promoter regions of *syrF* and *syrG*; and define the regulatory interactions of *salA*, *syrF*, *syrG*, and the promoter region of syringomycin biosynthesis genes.

Elucidation of the promoter regions of *syrF* and *syrG* was achieved transcriptional analysis, GFP reporters, and sequence analysis to identifiy common characteristics of the promoter regions of *syrF*, and *syrG*. It was revealed that the promoter sequences of *syrG* and *syrF* were highly similar to each other, but were distinctly different from the promoter region of *salA*. Alignment of these promoter sequences also identified a conserved sequence observed around the -35 region of the promoter. It was hypothesized that these conserved sequences are the binding site for SalA. Previous studies by Lu et. al (19) showed that SalA is required for the functional activation of *syrG* and *syrF*. In addition, Wang et. al (70) established that SalA binds to the promoter region of SyrF to activate transcription. It is unknown if similar conserved sequences observed around the -35 promoter region of *syrG* and *syrF* is found in the promoter region of *sylA*, given that *sylA* is under the transcriptional control of SalA (21). It has been shown that the promoters of related genes, under the control of a LuxR regulatory protein, display conserved promoter sequences of the promoter regions that

encode transcription factor binding sites for sigma factors and/or transcriptional regulators (23, 65, 69). Wang et. al (23) identified a conserved 20-bp sequence around the -35 region promoter region in syringomycin and syringopeptin biosynthesis genes, referred to as the *syr-syp* box. This *syr-syp* box was not identified in the promoters of *syrG* and *syrF*, indicating SalA has a binding site distinctly different from the *syr-syp* box. It is believed that SalA coordinates the expression of syringomycin and syringopeptin biosynthesis genes indirectly by binding to the promoters regions of *syrG* and *syrF*. It was established that SyrF forms dimers that recognize the *syr-syp* box as a binding site (22, 23). It is still unknown if SyrG recognizes the *syr-syp* box as a putative binding site. Similar binding sites have been identified in the promoters of genes regulated by LuxR's. In *Vibrio fischeri*, the gene that encodes LuxR in binds to a 20-bp sequence with dyad symmetry called the *lux* box which is located at the -42.5 position relative to the transcriptional start site of *luxI* and activates transcription of the *lux* operon (22, 65). A similar sequence with dyad symmetry was identified for the binding site of GerE in *Bacillus subtilis* (41). In addition, a *las-rhl* box was identified in the promoters of genes controlled by quorum sensing in *Pseudomonas aeruginosa* (21).

As reported in chapter II, the genes that encode the LuxR-like proteins SalA, SyrG, and SyrG are required for syringomycin production. It was demonstrated that SyrG is important for the transcriptional regulation of syringomycin and could potentially be in competition with SyrF for the same transcription factor binding site in the promoters of *syr-syp* genes. There was a strong indication that the interaction between SalA, SyrG, and SyrF for the regulation of syringomycin was complex, which

was studied in this chapter. The specific aim was to further define the regulatory interactions of SalA, SyrF, and syrG with the promoters of *salA*, *syrF*, *syrG*, and *syrB1*. GFP reporter assays were used to determine the functional activity of promoters transcriptionally fused to *gfp* in *Pss* B728a mutant derivatives. A similar study was performed by Ramel et. al, (21) that showed that the promoters of syringolin biosynthesis genes require *sylA* for activation. These GFP assays showed that SalA is an upstream transcriptional activator of *syrG* and *syrF*, SyrG an upstream positive regulator of *syrF* promoters, and both SyrG and SyrF are involved in the transcriptional activation of the syringomycin biosynthesis gene *syrB1*. In addition, the overexpression of *syrF* was able to restore syringomycin production in *syrG* derivative mutants. In contrast, the overexpression of *syrG* was not able to restore syringomycin production in *syrF* derivative mutants. These results confirmed that SyrG is indeed located upstream of SyrF in a regulatory cascade that encompasses SalA, SyrG, and SyrF. The effect of SyrG on the promoters of syringomycin biosynthesis genes may be indirect given that previous experiments performed by Wang et. al (22) has established the SyrF binds to the promoter of *syrB1* where there is no evidence to support that SyrG binds directly to these promoter regions. However, the data represented in this study does support the hypothesis that both SyrF and SyrG may be in compepititon for the same binding site found in the promoters of syringomycin biosynthesis genes based on overexpression analysis and GFP reporter assays.

The interactions observed between SalA, SyrG, and SyrF in the regulation of syringomycin and syringopeptin production by Pss B728a is complex. In the genome of

Pss B728a there are 24 genes that encode LuxR-like proteins, where *salA*, *syrG*, and *syrF* were the most highly expressed LuxR's in the apoplast (chapter II). Expression of *syrG* and *syrF* was a clear indication of the importance of these transcriptional regulators in virulence and pathogenicity. This study demonstrated that the SyrG and SyrF regulons overlap in regards to the regulation of syringomycin and syringopeptin production. In addition, both SyrG and SyrF exhibit highly similar promoters where SalA binds to activate transcription. Both SyrG and SyrF activate promoters of syringomycin biosynthesis genes where SyrG is an upstream transcriptional activator of *syrF*. In contrast, SyrG and SyrF were not involved in the regulation of virulence and metabolite genes that reside outside of the *syr-syp* gene cluster. The interactions observed between SalA, SyrG, SyrF, and *syr-syp* genes led to the conclusion that the regulation of syringomycin and syringopeptin is complex and it likely plays a role in the plant-pathogen interaction.

CHAPTER IV

CONCLUSIONS

P. syringae pv. *syringae* B728a is an economically important plant pathogen that utilizes a variety of sigma factors including (ECF) sigma factors and transcriptional regulators to coordinated the expression of genes in response to environmental signals (45, 47, 55, 69). LuxR regulatory proteins play an important role in the transcriptional regulation of a variety of biological processes involving two-component signaling, quorum sensing, and secondary metabolism. Analysis of the *Pss* B728a genome identified 24 LuxR-like proteins, three of which are *salA*, *syrG*, and *syrF* located adjacent to the syringomycin gene cluster. Previous studies implicated salA, syrG, and syrF in syringomycin production and virulence (19), however the regulatory role in regards to syringomycin production and the production of other secondary metabolites was not defined. In this study I addressed the hypothesis that the LuxR-like protein SyrG in involved in the regulation of genes essential for the pathogenic lifestyle of *Pss* B728a.

Bioinformatic analysis of the LuxR-like proteins found in the genome of Pss B728a revealed that the LuxR's were placed into four subfamilies based on domain architecture and the mechanism of regulatory activation. The LuxR-like proteins encoded by *salA*, *syrG*, and *syrF* were placed in a subfamily of LuxR's that were associated with secondary metabolism. It was interesting to discover that both *syrG* and *syrF* were the most highly expressed LuxR's in the apoplast of bean. These results led to

the initial hypothesis that SyrG was a transcriptional regulator of genes critical to plant pathogenesis. Pathogenicity assays showed that deletion mutants of syrG and syrF displayed a reduction in virulence by 80% and 50%, respectively. The double deletion mutant strain of Pss B728a that lacked syrG and syrF displayed disease symptoms that were comparable to the syrG mutant. Mutants of syrG and syrF did not have an effect on the bacterium's ability to replicate *in planta*. This dissertation confirmed that *syrG* and *syrF* were an important transcriptional regulator of genes associated with the biosynthesis of syringomycin and syringopeptin but were not involved in the regulation of virulence genes that reside outside of the *syr-syp* gene cluster. The data obtained from this study indicated that *syrG* and *syrF* were involved in overlapping regulons that are involved in the regulation of syringomycin production. There was a strong indication that the interactions between SalA, SyrG, and SyrF for the regulation of syringomycin and syringopeptin were complex.

Overexpression analysis and GFP fusion constructs were used to evaluate the interaction of SalA, SyrG, and SyrF in the regulation of syringomycin and syringopeptin biosynthesis genes. My research confirmed that SyrG is an upstream transcriptional activator of syrF, where both SyrG and SyrF activate the promoters of syringomycin biosynthesis genes. Also the promoters of syrG and syrF were highly similar to each other with conserved sequences observed around the -35 promoter region. It was hypothesized that these conserved sequences are the binding site for SalA. Previous studies by Wang et. al (22) established that SalA binds to the promoter region of SyrF to activate transcription. The interactions observed between SalA, SyrG, SyrF, and *syr-syp*

genes led to the conclusion that the regulation of syringomycin and syringopeptin is very complex and it likely plays a role in the plant-pathogen interaction.

REFERENCES

1. **Hirano SS, Upper CD.** 1990. Population biology and epidemiology of *Pseudomonas syringae*. Annu. Rev. Phytopathol. **28:**155-177.

2. **Bender CL, Alarcon-Chaidez F, Gross DC.** 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol. Rev. **63:**266-292.

3. **Feil H, Feil WS, Chain P, Larimer F, DiBartolo G, Copeland A, Lykidis A, Trong S, Nolan M, Goltsman E, Thiel J, Malfatti S, Loper JE, Lapidus A, Detter JC, Land M, Richardson PM, Kyrpides NC, Ivanova N, Lindow SE.** 2005. Comparison of the complete genome sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. Proc. Natl. Acad. Sci. U. S. A. **102:**11064-11069.

4. **Arrebola E, Cazorla FM, Perez-García A, de Vicente A.** 2011. Chemical and metabolic aspects of antimetabolite toxins produced by *Pseudomonas syringae* Pathovars. Toxins (Basel) **3:**1089-1110.

5. **Hirano SS, Rouse DI, Clayton MK, Upper CD.** 1995. *Pseudomonas syringae* pv. *syringae* and bacterial brown spot of snap bean: a study of epiphytic phytopathogenic bacteria and associated disease. Plant Dis. **79:**1085-1093.

6. **Gross DC, Cody YS, Proebsting E, Radamaker GK, Spotts RA.** 1984. Ecotypes and pathogenicity of ice-nucleation-active *Pseudomonas syringae* isolated from deciduous fruit tree orchards. Phytopathology **74:**241-248.

7. **Monier JM, Lindow SE.** 2004. Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. Appl. Environ. Microbiol. **70:**346-355.

8. **Monier JM, Lindow SE.** 2005. Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. Microb. Ecol. **49:**343-352.

9. **Laue H, Schenk A, Li H, Lambertsen L, Neu TR, Molin S, Ullrich MS.** 2006. Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. Microbiology **152:**2909-2918.

10. **Beattie GA, Lindow SE.** 1994. Epiphytic fitness of phytopathogenic bacteria: physiological adaptations for growth and survival. Curr. Top. Microbiol. Immunol. **192:**1-27.

11. **Hirano SS, Upper CD.** 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*- a pathogen, ice nucleus, and epiphyte. Microbiol. Mol. Biol. Rev. **64:**624-653.

12. **Yu X, Lund SP, Scott RA, Greenwald JW, Records AH, Nettleton D, Lindow SE, Gross DC, Beattie GA.** 2013. Transcriptional responses of *Pseudomonas syringae* to growth in epiphytic versus apoplastic leaf sites. Proc Natl Acad Sci U S A **110:**E425-434.

13. **Baltrus DA, Nishimura MT, Romanchuk A, Chang JH, Mukhtar MS, Cherkis K, Roach J, Grant SR, Jones CD, Dangl JL.** 2011. Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. PLoS Pathog. **7:**e1002132.

14. **Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW.** 2011. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol. Rev. **35:**652-680.

15. **Studholme DJ.** 2011. Application of high-throughput genome sequencing to intrapathovar variation in *Pseudomonas syringae*. Mol. Plant Pathol. **12:**829-838.

16. **Gross H, Loper JE.** 2009. Genomics of secondary metabolite production by *Pseudomonas* spp. Nat. Prod. Rep. **26:**1408-1446.

17. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959-964.

18. **Kitten T, Kinscherf TG, McEvoy JL, Willis DK.** 1998. A newly identified regulator is required for virulence and toxin production in *Pseudomonas syringae*. Mol. Microbiol. **28:**917-929.

19. **Lu SE, Scholz-Schroeder BK, Gross DC.** 2002. Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of *Pseudomonas syringae* pv. *syringae*. Mol. Plant Microbe Interact. **15:**43-53.

20. **Lu SE, Wang N, Wang J, Chen ZJ, Gross DC.** 2005. Oligonucleotide microarray analysis of the *salA* regulon controlling phytotoxin production by *Pseudomonas syringae* pv. *syringae*. Mol. Plant Microbe Interact. **18:**324-333.

21. **Ramel C, Baechler N, Hildbrand M, Meyer M, Schadeli D, Dudler R.** 2012. Regulation of biosynthesis of syringolin A, a *Pseudomonas syringae* virulence factor targeting the host proteasome. Mol. Plant Microbe Interact. **25:**1198-1208.

22. **Wang N, Lu SE, Records AR, Gross DC.** 2006. Characterization of the transcriptional activators SalA and SyrF, Which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae*. J. Bacteriol. **188:**3290-3298.

23. **Wang N, Lu SE, Yang Q, Sze SH, Gross DC.** 2006. Identification of the *syr-syp* box in the promoter regions of genes dedicated to syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae* B301D. J. Bacteriol. **188:**160-168.

24. **Wang N, Lu SE, Wang J, Chen ZJ, Gross DC.** 2006. The expression of genes encoding lipodepsipeptide phytotoxins by *Pseudomonas syringae* pv. *syringae* is coordinated in response to plant signal molecules. Mol. Plant Microbe Interact. **19:**257-269.

25. **Scholz-Schroeder BK, Hutchison ML, Grgurina I, Gross DC.** 2001. The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. Mol Plant Microbe Interact **14:**336-348.

26. **Subramoni S, Venturi V.** 2009. LuxR-family 'solos': bachelor sensors/regulators of signalling molecules. Microbiology **155:**1377-1385.

27. **Yang M, Giel JL, Cai T, Zhong Z, Zhu J.** 2009. The LuxR family quorum-sensing activator MrtR requires its cognate autoinducer for dimerization and activation but not for protein folding. J. Bacteriol. **191:**434-438.

28. **Maris AE, Sawaya MR, Kaczor-Grzeskowiak M, Jarvis MR, Bearson SM, Kopka ML, Schröder I, Gunsalus RP, Dickerson RE.** 2002. Dimerization allows DNA target site recognition by the NarL response regulator. Nat. Struct. Biol. **9:**771-778.

29. **Zhang JH, Xiao G, Gunsalus RP, Hubbell WL.** 2003. Phosphorylation triggers domain separation in the DNA binding response regulator NarL. Biochemistry **42:**2552-2559.

30. **Kaplan HB, Greenberg EP.** 1987. Overproduction and purification of the *luxR* gene product: Transcriptional activator of the *Vibrio fischeri* luminescence system. Proc. Natl. Acad. Sci. U. S. A. **84:**6639-6643.

31. **Piper KR, Beck von Bodman S, Farrand SK.** 1993. Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. Nature **362:**448-450.

32. **Welch M, Todd DE, Whitehead NA, McGowan SJ, Bycroft BW, Salmond GP.** 2000. N-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in *Erwinia*. EMBO J. **19:**631-641.

33. **Hussain MB, Zhang HB, Xu JL, Liu Q, Jiang Z, Zhang LH.** 2008. The acyl-homoserine lactone-type quorum-sensing system modulates cell motility and virulence of *Erwinia chrysanthemi* pv. *zeae*. J. Bacteriol. **190:**1045-1053.

34. **Gambello MJ, Iglewski BH.** 1991. Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, a transcriptional activator of elastase expression. J. Bacteriol. **173:**3000-3009.

35. **Selin C, Fernando WG, de Kievit T.** 2012. The PhzI/PhzR quorum-sensing system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomonas chlororaphis* PA23. Microbiology **158:**896-907.

36. **Brint JM, Ohman DE.** 1995. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family. J. Bacteriol. **177:**7155-7163.

37. **Shadel GS, Baldwin TO.** 1992. Positive autoregulation of the *Vibrio fischeri luxR* gene. LuxR and autoinducer activate cAMP-catabolite gene activator protein complex-independent and -dependent *luxR* transcription. J. Biol. Chem. **267:**7696-7702.

38. **Wilson DJ, Xue Y, Reynolds KA, Sherman DH.** 2001. Characterization and analysis of the PikD regulatory factor in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*. J Bacteriol **183:**3468-3475.

39. **Danot O.** 2001. A complex signaling module governs the activity of MalT, the prototype of an emerging transactivator family. Proc Natl Acad Sci U S A **98:**435-440.

40. **He W, Lei J, Liu Y, Wang Y.** 2008. The LuxR family members GdmRI and GdmRII are positive regulators of geldanamycin biosynthesis in *Streptomyces hygroscopicus* 17997. Arch Microbiol **189:**501-510.

41. **Ducros VM, Lewis RJ, Verma CS, Dodson EJ, Leonard G, Turkenburg JP, Murshudov GN, Wilkinson AJ, Brannigan JA.** 2001. Crystal structure of GerE, the ultimate transcriptional regulator of spore formation in *Bacillus subtilis*. J. Mol. Biol. **306:**759-771.

42. **Subramoni S, Gonzalez JF, Johnson A, Pechy-Tarr M, Rochat L, Paulsen I, Loper JE, Keel C, Venturi V.** 2011. Bacterial subfamily of LuxR regulators that respond to plant compounds. Appl. Environ. Microbiol. **77:**4579-4588.

43. **Chen J, Xie J.** 2011. Role and regulation of bacterial LuxR-like regulators. J. Cell. Biochem. **112:**2694-2702.

44. **Patankar AV, González JE.** 2009. Orphan LuxR regulators of quorum sensing. FEMS Microbiol. Rev. **33:**739-756.

45. **Thakur PB, Vaughn-Diaz VL, Greenwald JW, Gross DC.** 2013. Characterization of five ECF sigma factors in the genome of *Pseudomonas syringae* pv. *syringae* B728a. PLoS One **8:**e58846.

46. **Greenwald JW.** 2011. Ph.D. dissertation. An In-depth Analysis of Iron and Pathogenicity Regulatory Networks in *Pseudomonas* syr*ingae pv. syringae* B728a. Texas A&M University, College Station, TX.

47. **Records AR, Gross DC.** 2010. Sensor kinases RetS and LadS regulate *Pseudomonas syringae* type VI secretion and virulence factors. J. Bacteriol. **192:**3584-3596.

48. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-6645.

49. **Marco ML, Legac J, Lindow SE.** 2005. *Pseudomonas syringae* genes induced during colonization of leaf surfaces. Environ Microbiol **7:**1379-1391.

50. **Miller WG, Leveau JH, Lindow SE.** 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. Mol. Plant Microbe Interact. **13:**1243-1250.

51. **Lu SE, Scholz-Schroeder BK, Gross DC.** 2002. Construction of pMEKm12, an expression vector for protein production in *Pseudomonas syringae*. FEMS Microbiol Lett **210:**115-121.

52. **Leong SA, Ditta GS, Helinski DR.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. J Biol Chem **257:**8724-8730.

53. **Vidaver AK.** 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. Appl Microbiol **15:**1523-1524.

54. **KING EO, WARD MK, RANEY DE.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J Lab Clin Med **44:**301-307.

55. **Greenwald JW, Greenwald CJ, Philmus BJ, Begley TP, Gross DC.** 2012. RNA-seq analysis reveals that an ECF σ factor, AcsS, regulates achromobactin biosynthesis in *Pseudomonas syringae* pv. *syringae* B728a. PLoS One **7:**e34804.

56. **Huynh TV, Dahlbeck D, Staskawicz BJ.** 1989. Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science **245:**1374-1377.

57. **Landy A.** 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu Rev Biochem **58:**913-949.

58. **Sawahel W, Sastry G, Knight C, Cove D.** 1993. Development of an electro-transformation system for *Escherichia coli DH10B*, p. 261-266, vol. 7. Biotechnology Techniques.

59. **Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS.** 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. **39:**D596-600.

60. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **3:**1101-1108.

61. **Quiñones B, Dulla G, Lindow SE.** 2005. Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. Mol Plant Microbe Interact **18:**682-693.

62. **Choi SH, Greenberg EP.** 1992. Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. J Bacteriol **174:**4064-4069.

63. **Hutchison ML, Gross DC.** 1997. Lipopeptide phytotoxins produced by Pseudomonas syringae pv. syringae: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. Mol Plant Microbe Interact **10:**347-354.

64. **Scholz-Schroeder BK, Soule JD, Gross DC.** 2003. The *sypA*, *sypB*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. Mol. Plant Microbe Interact. **16:**271-280.

65. **Finney AH, Blick RJ, Murakami K, Ishihama A, Stevens AM.** 2002. Role of the C-terminal domain of the alpha subunit of RNA polymerase in LuxR-dependent transcriptional activation of the *lux* operon during quorum sensing. J. Bacteriol. **184:**4520-4528.

66. **Notredame C, Higgins DG, Heringa J.** 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol **302:**205-217.

67. **Agaras B, Sobrero P, Valverde C.** 2013. A CsrA/RsmA translational regulator gene encoded in the replication region of a Sinorhizobium meliloti cryptic plasmid complements *Pseudomonas fluorescens rsmA/E* mutants. Microbiology **159:**230-242.

68. **Browning DF, Busby SJ.** 2004. The regulation of bacterial transcription initiation. Nat Rev Microbiol **2:**57-65.

69. **Saha S, Lindeberg M.** 2013. Bound to Succeed: Transcription Factor Binding-site Prediction and Its Contribution to Understanding Virulence and Environmental Adaptation in Bacterial Plant Pathogens. Mol Plant Microbe Interact **26:**1123-1130.

70. **Filiatrault MJ, Stodghill PV, Myers CR, Bronstein PA, Butcher BG, Lam H, Grills G, Schweitzer P, Wang W, Schneider DJ, Cartinhour SW.** 2011. Genome-wide identification of transcriptional start sites in the plant pathogen *Pseudomonas syringae* pv. *tomato* str. DC3000. PLoS One **6:**e29335.